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An Assessment of the Evolutionary Stability of Distyly in Hedyotis Caerulea (Rubiaceae)

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AN ASSESSMENT OF THE EVOLUTIONARY STABILITY OF DISTYLY IN *HEDYOTIS CAERULEA* (RUBIACEAE)

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ABSTRACT

Distylous species of flowers possess two distinct floral morphs that are generally found in equal numbers in naturally occurring populations. The flower form that has a relatively long style is called a "pin"; the form with a short style is a "thrum." Within the distylous mating system, selfing and intramorph mating are prevented due to the spatial separation of stigmas and anthers and by a self-incompatibility system that is inherited along with the dimorphic floral structure and ancillary polymorphisms. However, a breakdown of distyly has been documented in several families, either through the development of completely separate sexes (dioecy), or, more frequently, through the development of monomorphy and self-fertile homostyles.

Here several populations of *Hedyotis. caerulea*, a perennial spring-flowering herb native to eastern North America, were surveyed in the Cleveland Metroparks and sampled to investigate the degree of distyly present and to detect any movement of the mating system towards selfing or dioecy. Morphometric analysis of the variation within and across populations of stigma height and anther height indicate that this species exhibits reciprocal herkogamy. Nearly all populations surveyed were isoplethic (i.e., contained a morph ratio of pins to thrums that was not different from 1:1), which is found when the species' mating system promotes intermorph pollen transfer (dissortative

mating) with nearly 100% outcrossing. There was no indication that the self-incompatibility system has broken down with no movement towards homostyly.

Variation was present, however, in these primary traits as well as in stigma length, corolla tube width, pollen diameter, pollen count, dry weight, and seed set of the flowers collected among populations. Several ancillary floral traits, including corolla tube shape, pollen diameter, and length of stigmatic papilla, likewise varied. Across populations, the two morphs invested a similar amount of biomass in the production of pollen and seed, suggesting that each morph had equal male and female fitness with no movement towards dioecy. Given the significant degree of variation found in each morphometric analysis, extensive modification is possible were it favored by selection. Therefore the distylous mating system in *H. caerulea* appears to be a stable one.

Finally, the development of distyly was investigated by contrasting growth of the flower buds and the heights of anthers and stigmas within the bud prior to the flower opening. Anthers of the two morphs grew at different rates throughout development of the bud, but growth patterns of the stigma were more complex. Stylar growth began only slightly slower in thrum flowers than in pins, but growth decreased over time resulting in a curvilinear growth pattern for thrums.

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CHAPTER I

INTRODUCTION

The evolution of sex has been difficult to explain from a theoretical perspective. Charles Darwin and R. A. Fisher, pioneers in evolutionary theory, tackled the problem, and progress since has involved a synthesis of ideas from classical genetics, population genetics, molecular genetics, evolutionary ecology, behavioral ecology, and comparative biology (Charlesworth 2006). Sex in flowering plants is further complicated by three features of their reproductive biology. First, their structural simplicity allows most plants to reproduce asexually, without the fusion of gametes (Richards 1997). A plant with its own distinctive genotype (genet) can produce many physiologically independent individuals (ramets) through clonal growth, or vegetative reproduction. While most plant biologists do not consider clonal growth actual reproduction, some species are able to produce seeds parthenogenetically (apomixis). Second, the vast majority (approximately 95%) of flowering plants are hermaphroditic; i.e., they have both male and female sex organs (Richards 1997). Sperm cells develop within pollen grains in the male reproductive organs (anthers), and ovules develop within the female reproductive apparatus (the pistil). A flower, therefore, can function as both the maternal and paternal

parent when it reproduces. Further, if pollen is transferred from the anthers to the pistil within a single flower, self-fertilization may follow. In other words, a flower can be its own sex partner (Richards 1997). Third, since plants are sessile in any single generation, they cannot actively choose their sexual partners. In order to mate with other individuals, flowers rely upon vectors (such as insects, birds, wind, or water) to deliver pollen to the pistils of their potential sex partners (outbreeding). Since individual flowers typically produce vast quantities of pollen and multiple ovules, sex among plants can be extremely promiscuous. Individual plants will mate with a number of sexual partners chosen by the appropriate pollinators, and consequently, it is difficult to understand the mating system of a plant species without consideration of how the pollinators involved in that system spread gametes among mates (Richards 1997). These three features of angiosperm biology have given flowering plants a range of reproductive options, based upon three basic mechanisms of reproduction: apomixis, selfing, and outbreeding. Each of these reproductive options carries its own costs and benefits.

Apomixis increases a population's biomass without the expenditure of energy required to reproduce sexually. From an evolutionary perspective, the reproductive assurance of asexual reproduction (apomixis) constantly challenges the greater genetic variability and fitness afforded by sexual reproduction in flowering plants. In fact, the transition to complete asexuality is possible (e.g. *Taraxacum officinale*, a completely apomictic species of dandelion). However, since plants produced asexually are genetically identical to their parent plants, apomixis greatly limits the creation of new genetic variation upon which natural selection can act in the face of changing environmental conditions.

As in asexual reproduction, self-fertilization allows an isolated flower to reproduce independently with little expenditure of energy. However, in many species self-fertilized flowers produce fewer seeds, and the seeds produced are of lower quality than when outcrossed; i.e., they exhibit inbreeding depression. Darwin (1876) studied the consequences of inbreeding by quantifying reproductive success over several generations in a number of species (e.g. *Digitalis purpurea*, *Zea mays*, and *Ipomeoea purpurea*) and published his results in his book, *The Effects of Cross and Self Fertilisation in the Vegetable Kingdom*. He found that the progeny of cross-fertilized plants were generally superior in performance when compared with those self-fertilized, and he concluded that avoidance of self-fertilization has been an important selective pressure in plant evolution. Since Darwin, the consequences of inbreeding depression in plant populations have been investigated intensely (as reviewed in Wright 1977; Charlesworth and Charlesworth 1993; Thornhill 1993; Keller and Waller 2002). However, while outbreeding avoids inbreeding depression, it carries greater energy costs. Compared with species that self-fertilize regularly, obligate outcrossers must produce a greater amount of biomass to create reproductive structures (i.e., flowers, pollen, and nectar), much of which provides no return with respect to increasing offspring numbers to the next generation. Perhaps a greater consequence of relying exclusively on outcrossing is a dependence on pollinators. If the number of pollinators dwindles, so might the opportunity for sexual reproduction in any pollinator-dependent plant species. Such a scenario could lead to diminished seed set, diminished population size, and, to the extreme, possible extinction. Thus, reproduction in obligate outcrossers is not as assured as it is with selfing and apomixis.

The majority of plant species predominately outcross, and most rarely, if ever, self-fertilize. While some 40% of plant taxa self at various times (Richards 1997), only 20–25% of plant taxa self predominately (Barrett and Eckert 1990). For example, most of the world's most important crops (e.g., peas, rice, beans, corn, and wheat) are primarily self-fertilizing (Brown 1990). Since both selfing and outcrossing have potential benefits and costs that vary relative to environmental conditions and the availability of pollinators, it is not surprising that many flowering plants employ a reproductive strategy called mixed mating that includes selfing and outcrossing in varying proportions. In some species, the percentage of offspring created by outcrossing is very low, while in others it exceeds 50 %. Halliburton (2004, p. 287) quantified the theoretical relationship between selfing and inbreeding with a recursion equation for the inbreeding coefficient in a system with mixed self-fertilization and random mating as

$$f_{t+1} = \tfrac{1}{2} S (1 + f_t)$$

where f is the inbreeding coefficient, S is the proportion of self-fertilization, and t is generation. Thus, this system would promote outcrossing by minimizing S.

However, in a pair of controversial papers (one theoretical, the other empirical), Lande and Schemske (1985) and Schemske and Lande (1985) argued that evolutionary stability can only be reached in a system in which there is either complete outcrossing or complete selfing. They reasoned that if even partial selfing occurs in a population, recessive deleterious alleles would be purged rapidly. They supported their theoretical argument with a survey of 55 plant species in which they analyzed estimates of the proportion of seeds produced through outcrossing for each taxon. They found a bimodal frequency distribution which they argued was consistent with their prediction for only

two stable endpoints of mating system evolution: predominant outcrossing with strong inbreeding depression and predominant selfing with weak inbreeding depression. However, Holsinger (1992) developed a mathematical model that better reflected the empirical evidence, and he showed that mixed mating strategies may often be evolutionarily stable.

The growing body of empirical evidence collected since 1985 has contradicted Lande and Schemske's position. Numerous plant species with mixed mating systems that appear to be evolutionarily stable have been identified (Waller 1986; Aide 1986; Barrett and Eckert 1990). Species with a genuine mixed mating system can achieve intermediate and often variable amounts of outcrossing through a mixture of self- and cross-pollination at various levels. For example, in plants such as *Viola canina* (Darwin 1877), *Impatiens capensis* (Schemske 1978), and *Laminium amplexicaule* (Lord 1979, 1982), individuals produce developmentally distinct self-pollinated (cleistogamous) and open-pollinated (chasmogamous) flowers that clearly maintain an intermediate selfing rate. Further, when Goodwillie et al. (2005) expanded the Schemske and Lande (1985) survey to include 345 species in 78 families, they did not observe the bimodal distribution identified in the original survey, presumably because the number of predominantly selfing taxa had been overestimated in the earlier survey.

Characterization and classification of plant mating systems remains controversial. For experimental purposes Brown (1990) has divided plant species into five classes of mating: (1) predominately outcrossing (i.e., self-fertilization rate, $s < 0.05$); (2) predominantly self-fertilizing (i.e., outcrossing rate, $t < 0.10$ (Fryxell 1957); (3) mixed selfing and outcrossing; (4) partially apomictic; and (5) partial selfing of the

gametophytes. However, Jain (1984) argued that plant mating systems should be thought of as a continuum of possibilities rather than of strict types.

While little is understood about how plants "know" when to self and when to outcross, several strategies have evolved in flowers that reduce the likelihood of self-pollination, including dichogamy (maturation of male and female reproductive organs at different times), herkogamy (spatial separation of reproductive organs), and dioecy (complete separation of reproductive organs on male and female flowers). Once pollination has occurred, many flowers are able to prevent self-fertilization by rejecting pollen produced by the same flower or individual; these flowers are said to express self-incompatibility (SI).

Self-Incompatibility

De Nettanourt (1977) defined SI as the "inability of a fertile hermaphrodite seed plant to produce a zygote after self-pollination." SI is a genetically controlled recognition and rejection process that biochemically prevents self-fertilization. In a "compatible" pollination, pollen grains (which contain sperm cells) land on and adhere to the stigma (the distal end of the pistil). The pollen grains hydrate and germinate, forming a pollen tube (the sperm-delivery apparatus), which grows through the stigma and style (a tube between the stigma and the ovary of the pistil) in a tract of transmitting tissue until it reaches the ovary where an ovule is fertilized. When a flower is selfed, however, the pollination is "incompatible" because the pollen tube either fails to germinate or its growth is aborted somewhere along its path to the ovary. Thus, when the pollen and pistil are of the same type, interactions between the pistil of the maternal plant and the pollen from the paternal plant result in the inhibition of pollen tube growth.

A variety of SI systems have evolved in flowering plants that use distinctive mechanisms for both pollen recognition and pollen tube inhibition. Since different SI systems are found in various plant families, SI is believed to have evolved independently multiple times (Nasrallah 2005).

SI systems consist of two phases: a recognition phase, in which pollen is identified as “self” or “non-self”, and a response phase, in which self-pollen is rejected. The recognition phase is regulated by a linked cluster of genes collectively known as the "S-locus". Individual plants that share recognition haplotypes at the S-locus are incapable of successfully producing offspring (Richards 1997; Busch and Schoen 2008) because their pollen is rejected during the response phase. Pollen rejection is triggered during recognition phase and is regulated by genes outside the S-locus. While all SI systems share these two phases, the functions are accomplished by significantly different mechanisms. In fact two drastically different genetic recognition systems and three distinct response mechanisms have been characterized.

Genetic Systems of Control of SI

Genetic control of pollen recognition may be gametophytic or sporophytic. In gametophytic systems, the incompatibility type of a pollen grain is determined by its own genotype. In sporophytic systems the incompatibility type of a pollen grain depends on the genotype of the plant that produced the pollen (Sims 1993).

Gametophytic self-incompatibility (GSI), found in the Solanaceae (nightshade and tobacco family), the Rosaceae (rose family), and the Papaveraceae (poppy family), is more widespread than sporophytic self-incompatibility (SSI) (Sims 1993). In GSI, if an individual plant is heterozygous for a gene *S,* the cells in the tissues that make up the

pistil are S_1S_2. Each sperm cell receives one of the two *S* haplotypes, either S_1 or S_2. After pollination, the pistil responds to pollen in accordance with the genotype of the pollen. If neither *S* haplotype in the genotype of the pistil matches an *S* haplotype of the pollen, the cross is compatible, a pollen tube is allowed to grow, and an ovule is fertilized. If the *S* allele of the pollen matches either haplotype of the pistil's genotype, the cross is incompatible, and pollen tube growth is aborted. The SI reaction arises from the gene products of pollen and style, permitting pollen tube growth in cross-pollinations and preventing pollen tube growth in self-pollinations (Sims 1993). By GSI, when a flower is selfed, the pollen would always be rejected in plants with a fully functional SI system.

The second system, sporophytic self-incompatibility (SSI), occurs in the Brassicaceae (cabbage family) and the Asteraceae (aster family) (Charlesworth 2000). In SSI, pollen is treated as if it had the genotype of its paternal plant. Individual plants that are heterozygous for gene *S* would have somatic cells with the genotype S_1S_2, and individual sperm cells with either an S_1 or an S_2 haplotype. When these flowers produce pollen, they manufacture recognition proteins encoded by both haplotypes and export them to the developing pollen walls (Sims 1993). During pollen-pistil interactions following pollination, the pistil responds to pollen as if it had the S_1S_2 genotype (the same as that of the paternal plant). When the maternal and paternal parents share any *S* allele in common, the pollination is incompatible. For example, pollen (haplotype S_1) from one S_1S_2 parent would be rejected by a pistil with genotype S_2S_3. Although the pollen (S_1) and the pistil (S_2S_3) do not share a common allele, the pollen carries S_2 recognition

proteins. As in GSI, incompatible reactions are characterized by the abortion of pollen tube growth.

Molecular Mechanisms of Pollen Rejection

At a molecular level, the SI specificity locus defines self and non-self. In a pistil pollinated by a mixture of pollen grains or tubes, those with non-self genotypes are selected to grow while those with self genotypes are inhibited. Various plant families use distinct mechanisms for pollen, which is consistent with phylogenetic evidence that suggests that SI has evolved independently multiple times during the diversification of flowering plants. GSI has been intensively studied at the molecular level in the Solanaceae (including genera such as *Solanum*, *Lycopersicon*, *Petunia*, *Nicotiana* and the *Papaveraceae)*. SSI has been studied largely in the *Brassicaceae*. Researchers have identified three different molecular mechanisms of SI to date: the mechanism found in the Brassicaceae (which is under sporophytic control), the mechanism found in the Solanaceae (which is under gametophytic control), and the mechanism found in the Papaveraceae (which is also under gametophytic control). Since SI in a large number of plant families has not yet been studied, additional mechanisms may exist.

In the Brassicaceae, the SI mechanism disrupts hydration and germination of incompatible pollen grains within minutes of their contact with the stigma. The *S*-locus codes for two highly polymorphic proteins (Kachroo et al 2002), a type of surface receptor on the pistil cells and a ligand released by the pollen grains. The receptor is the *S*-locus receptor kinase (SRK), a large membrane-bound receptor kinase found on the plasma membranes of the papilla cells on the surface of the pistil (Takayama and Isogai 2003). The ligand is a small, soluble protein called the S-locus cysteine-rich (SCR)

protein (Schopfer 1999), alternately referred to as SP11 (Takayama et al. 2000), which is released from the surface of the pollen grain when it comes in contact with a papilla cell. Together, SRK and SCR/SP11 form a receptor-ligand pair that determines SI specificity in stigma and pollen. When SCR/SP11 binds to the receptor domain of the SRK of a matching *S* haplotype, the SRK kinase domain is activated (Takayama et al 2001) and an intracellular signaling cascade is initiated that results in increased production of proteins in the papilla cell wall that prevent further development of the pollen grain or tube (Stone et al. 2003).

In the Solanaceae (as well as the Scrophulariaceae (snapdragons) and the Rosaceae), pollen inhibition is triggered within the pollen tube, rather than within the cells of the pistil, and self-pollen tubes are destroyed as they grow within the style. In this mechanism, the *S*-locus codes also for two polymorphic proteins: a ribonuclease secreted by the pistil (*S*-RNase) and an F-box protein (SLF) produced by the pollen (Sijacic et al. 2004). *S*-RNases enter pollen tubes nonselectively and act as cytotoxins, degrading the RNA needed for protein translation, and leading to pollen tube inhibition unless they are targeted for destruction by SLF through the attachment of ubiquitin. *S*-RNases from nonmatching *S* haplotypes are destroyed, leaving *S*-RNases from matching *S* haplotypes intact and able to inhibit pollen tube growth (Lai et al. 2002).

The third mechanism, found in the Papaveraceae, destroys self-pollen tubes as well but through a programmed cell death pathway. The stigma secretes a small extracellular chemical signal – a polymorphic glycoprotein encoded by the *S*-locus – that interacts with a receptor on pollen tube surfaces (Thomas and Franklin-Tong 2004). While it is clear that a protein made by the pollen is involved, that protein has not been

identified as yet. In this mechanism, self-stigma *S* proteins cause a rapid increase in Ca^{+2} within growing pollen tubes, characteristic of a programmed cell death (Thomas and Franklin-Tong 2004). As with the two previous mechanisms, SI in the poppy family demonstrates a high degree of recognition specificity.

Heterostyly

In populations of some flowering plant species, individuals fall into two or three morphologically distinct mating types, which differ in stigma height, anther height and several ancillary pollen and stigma polymorphisms (Darwin 1877; Vuilleumier 1967; Ganders 1979; Barrett 1992). This condition is known as distyly when two floral morphs exist, tristyly when there are three, and heterostyly when referring to the general condition. The floral morphs coexist at roughly equal frequencies within a population (Richards 1997). The relative positions of anthers and stigmas distinguish one morph from another. In distylous species, one morph is long-styled with short anthers ("pin"), and the other is short-styled with long anthers ("thrum"). An individual plant produces either pin flowers or thrum flowers – never both. Usually the thrum is heterozygous for the mating system chromosome *Ss*, whereas the pin is homozygous *ss* (Richards 1997). The defining feature of heterostylous populations is the arrangement of anthers and stigmas at corresponding positions within the corolla tube, also known as reciprocal herkogamy (Webb and Lloyd 1986).

Early in his work on heterostyly, Darwin (1877) conducted pollination studies in the cowslip (*Primula veris)*, a distylous species. When he transferred pin pollen to thrum stigmas and thrum pollen to pin stigmas, the plants produced good seed set. He considered these crosses "legitimate". When he self-pollinated pins or thrums, and when

he transferred pin pollen to the stigma of a different pin flower or thrum pollen to a different thrum flower, the plants produced many fewer seeds. He called these crosses "illegitimate" (see Fig 1.1). Darwin concluded that the two floral morphs represent two distinct mating types that are cross-compatible but incompatible within the same morph. He expanded his pollination studies to other heterostylous species, and compiled results of such crosses from other researchers, eventually publishing a monograph on the subject: *The Different Forms of Flowers on Plants of the Same Species* (Darwin 1877). To a large extent, studies of heterostyly since Darwin have focused on a few taxa (e.g. *Primula* and *Lythrum*) originally studied by Darwin (1877). Of these, *Primula* is the best studied group and is often represented in scientific literature as a model system for heterostyly (Mast and Conti 2006).

Darwin proposed that the reciprocal placement of anthers and stigmas was a mechanism to promote cross-pollination between floral morphs. According to his hypothesis, pollinators visiting heterostylous flowers pick up pollen on different parts of their bodies, thus promoting cross-pollination transfer between floral morphs. He further proposed that reciprocal herkogamy promotes efficiency in cross-pollination by reducing male gamete wastage on incompatible stigmas (Lloyd and Webb 1992a and b). Darwin's cross-pollination hypothesis has found considerable support by modern researchers (Kohn and Barrett 1992; Lloyd and Webb 1992b), and heterostyly is generally described as an outcrossing mechanism.

While reciprocal herkogamy has been referred to as the "emblem" of heterostyly (Dulberger 1992), two additional elements are commonly found in heterostylous species: (1) a diallelic, sporophytic self-incompatibility system exists that interrupts pollen tube

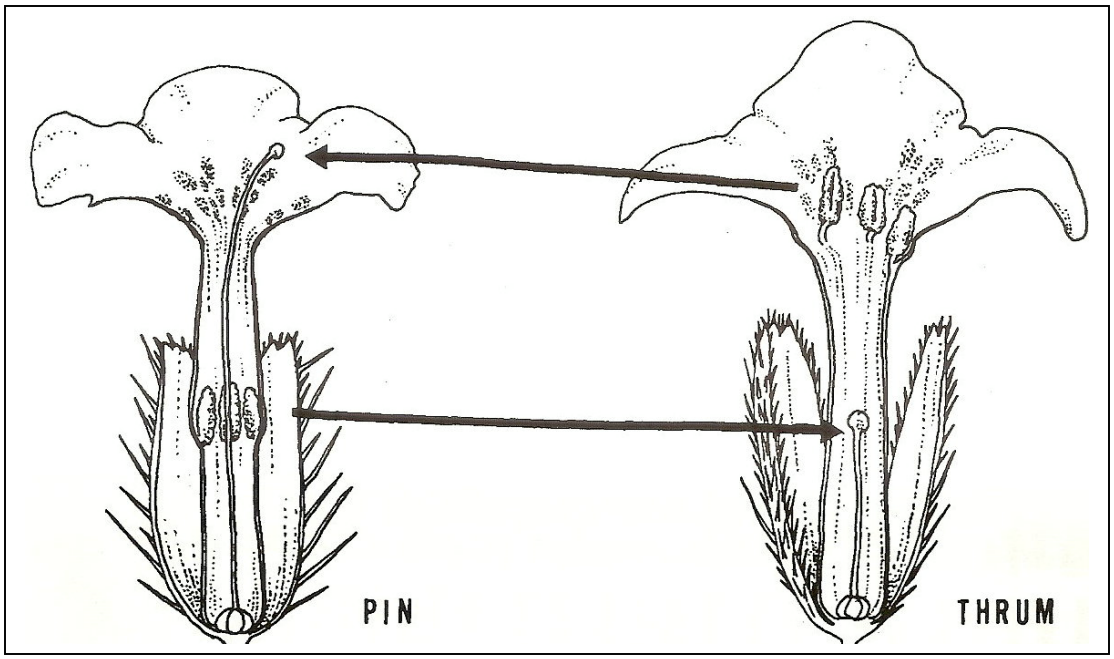

Fig. 1.1 Legitimate pollination between distylous flowers. Pollen is transferred from anthers to stigmas of equivalent height. The pin has a long style and short anthers, while the thrum has a short style and long anthers. (Source: Darwin 1877)

growth in an illegitimate cross (self or intramorph); and (2) a set of ancillary morphological polymorphisms of the stigmas and pollen of the floral morphs. Taken together, these three characteristics are referred to as the "heterostyly syndrome".

While the self-incompatibility system in heterostyles is sporophytic (Dulberger 1992), pollen rejection in heterostylous species is substantially different from that in homostylous species with sporophytic self-incompatibility (Gibbs 1986; Barrett and Cruzan 1994). Homostylous sporophytic self-incompatibility is characterized by pollen tube inhibition at a single site (Barrett and Cruzan 1994), but heterostylous sporophytic self-incompatibility involves pollen tube inhibition by various mechanisms, including a lack of adhesion, hydration, and germination of pollen, an inability of pollen tubes to penetrate the stigma surface, and an inhibition of pollen tube growth in the style and ovary (Bawa and Beach 1983; Wedderburn and Richards 1990; Dulberger 1992). In many of the species there appears to be a series of barriers that screen for illegitimate pollen tubes, none of which are completely successful alone. Together, however, they gradually eliminate illegitimate pollen tubes in a "cascade system" of pollen rejection (Shivanna et al. 1981, 1983).

The ancillary floral polymorphisms found in heterostyly include variation in pollen size, pollen color, pollen sculpturing, stigma shape, size of stigmatic papillae, and possession of hairlike projections within the corolla tubes (Ganders 1979; Dulberger 1992). The range of these polymorphisms varies among species (Dulberger 1992). No specific list of such characters found in all heterostylous species has been identified (Massinga et al. 2005), and the adaptive significance of these characters remains unclear.

The three components of heterostyly are believed to be controlled by a block of three tightly-linked loci, sometimes called a "supergene" – a model proposed by Ernst in 1955 (as cited in Lewis and Jones 1992) to explain the inheritance of distyly in *Primula*. The gene responsible for the mechanism of SI is thought to be linked to the genes responsible for reciprocal herkogamy and the ancillary floral polymorphisms (Barrett 1992; Lewis and Jones 1992). According to the supergene model, the *G* locus determines characteristics of the pistil (or gynoecium), including style length and the pistil's incompatibility response, *P* determines pollen size and the pollen's incompatibility response, and *A* determines anther height. The three dominant alleles at each locus are linked and make up the '*S*-allele' (*GPA*), while the three recessive alleles comprise the 's-allele' (*gpa*) (Lewis and Jones 1992). This genetic system is considered to be one of the best examples of a coadapted linkage group in plants (Wedderburn and Richards 1990).

Molecular Studies of Heterostyly

We know almost nothing about the molecular mechanisms responsible for heterostyly (Barrett et al. 2000). While GSI and homostylous SSI have been studied extensively at the molecular level (see reviews by Sims 1993 and Charlesworth et al. 2005), only a handful of studies have employed molecular techniques to explore heterostylous self-incompatibility.

The initial studies of heterostyly focused on distylous species of *Turnera* (Turneraceae). Athanasiou and Shore (1997) found three proteins unique to the styles and stigmas of the thrums, and mapped two izozyme loci to a region spanning the distyly locus, thus providing molecular evidence for the linkage of the genes for floral

polymorphisms and SI. Later, the same group described a polygalacturonase that localized to the style transmitting tissue of thrums of distylous *Turnera subulata* (Athanasiou et al. 2003). Tamari and Shore (2004) also detected a polygalacturonase specific to the thrums of six other distylous species of *Turnera.* Khosravi et al. (2004) detected a second thrum-specific protein, an alpha-dioxygenase, in five distylous species of *Turnera*. However, their specific roles in the expression of distyly are yet unknown.

The distylous species buckwheat, *Fagopyrum esculentum* (Polygonaceae) was the second heterostylous taxon to be studied at the molecular level. Miljus-Dukic et al. (2004) detected two distinct proteins specific to thrum pistils (as in *Turnera*). However, he also found a distinct group of proteins in *Fagopyrum* pin pistils. While the function of these proteins has not yet been demonstrated, the existence of a group of proteins suggests that the genes responsible must be either physically linked (which could be detected through genetics) or linked by regulating mechanisms. Variation in the proteins employed suggest that SSI in heterostyles may have evolved independently.

Evolution of Heterostyly

Lacking much information on how heterostyly works, the evolutionary origins of heterostyly have remained obscure. Especially necessary is comparison to the sister groups of distylous taxa. While substantial databases have been collected on characters, such as chromosome numbers and pollen morphology, plant mating systems have been largely ignored when describing new species, and such information has not been collected in any systematic way (Barrett et al. 2000).

Heterostyly is known in 28 animal-pollinated angiosperm families, including both monocots and dicots (Barrett 2002). However, heterostylous plants are still the minority

within the angiosperms. Ganders (1979) lists 155 genera that include heterostylous species, which constitute 1 – 2% of the genera of flowering plants. Supporting the biochemical evidence, the phylogenetic distribution of these families suggests that heterostyly has evolved independently at least 23 different times (Ganders 1979; Lloyd and Webb 1992a). Since heterostyly appears to have evolved multiple times in some families based on non-heterostylous sister groups, this number is most likely an underestimate.

Two quantitative models have been proposed to explain the origin of heterostyly: the Charlesworths' (1979) selfing avoidance model and the Lloyd and Webb (1992a) pollination efficiency model. The essential difference between them is order in which reciprocal herkogamy and SI first evolved. In Charleworths' model, SI evolved first, and then reciprocal herkogamy, in part because many botanists believe that self-incompatibility is an ancestral condition in the evolution of flowering plants. If SI evolved first, conspecific homostyles and heterostyles would likely share the same mechanism of SI. According to the Lloyd and Webb model (1992a), reciprocal herkogamy evolved first, and then SI (as Darwin had postulated). They reason that, if heterostyly is primarily an outcrossing mechanism, and if the ancestors of heterostylous species were already self-incompatible, the polymorphisms would not have been needed (Lloyd and Webb 1992a). While each of these theoretical models has relatively weak empirical support at the family level, a recent historical reconstruction of character evolution in the genus *Narcissus* supports the Lloyd and Webb model (Graham and Barrett 2004).

The Study Species

H. caerulea L. is a little studied distylous spring flowering herb native to North America. Commonly known as "bluets", these small, delicate perennials are noticeable only during their brief flowering period from the end of March until early June, when they appear to retreat to a dormant state underground until the following spring. They tend to grow in open shade and well-drained soil, always with moss, in a variety of semi-disturbed habitats such as forest margins, roadsides, and fields (personal observations). This species ranges from eastern Canada to the southeastern United States (Terrell 1996). Bluets tend to grow in compact tufts up to 20 cm tall. Given sufficient sunlight, the individual plants can spread vegetatively through rhizomes and fibrous roots, creating broad, circular patches. The foliage is a distinct rosette at the base of the plant, with several stems usually arising from a single base. Located on the ends of the stems, the flowers are light blue or occasionally white with a tubular corolla, four petal lobes, and a yellow center (Terrell 1996). Their major pollinators are bee flies (*Bombylius* sp.) and small native bees (Wyatt and Hellwig 1979; Grimaldi 1988; personal observations).

Distyly was noted in this species in 1862 by Asa Gray (Gray, 1862, letter to Charles Darwin, Cambridge University Library; Ornduff 1977), and Darwin included *H. caerulea* is his monograph on distyly (Darwin 1877). As with other distylous species, *H. caerulea* produces two distinct mating types, pin and thrum, which differ not only in the relative heights of their stigmata and anthers (see Fig. 1.2), but in ancillary floral characters, as well. Pins have small pollen grains and long stigmatic papillae, while thrums have large pollen grains and short stigmatic papillae (Ornduff 1977, 1980; Wyatt and Hellwig 1979; Terrell 1996; personal observations). It is also typical of a distylous

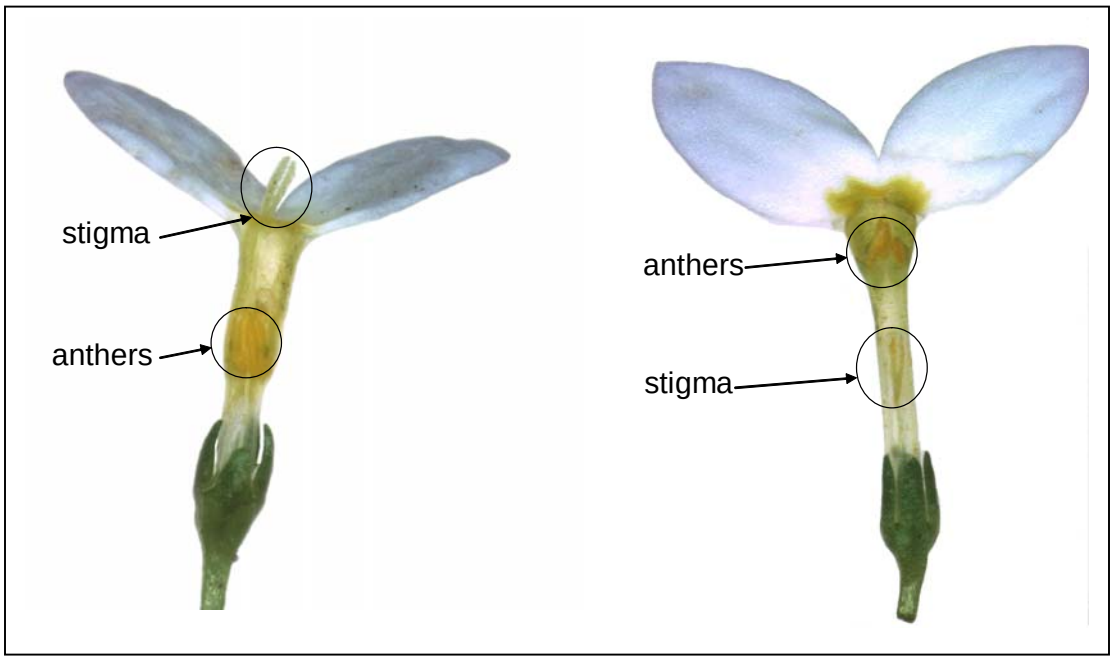

Fig. 1.2 Pin and thrum morphs of *H. caerulea.* The pin morph (on the left) has a long style that holds the stigma high and anthers positioned low in the corolla tube, while the thrum (on the right) has a short style that holds the stigma and anthers positioned high in the corolla tube.

species in that it requires insect pollinators to carry pollen between different morphs. Previous studies have demonstrated that intramorph crosses or self-pollinations produced little or no seed and intermorph crosses produced copious seed (Wyatt and Hellwig 1979; Ornduff 1977, 1980), suggesting that *H. caerulea* has a strong SI system.

Hedyotis is a member of the large, mostly tropical family Rubiaceae which includes as many as half of the genera of flowering plants that are heterostylous (Bir Bahadur, 1968; Vuilleumier, 1967). Bir Bahadur (1968) lists 416 distylous species in 91 genera of the Rubiaceae. Since these species are distributed among 21 different tribes and often have homostylous congeners (e.g. *Hedyotis*, Ornduff, 1969; *Pentas*, Verdcourt 1953), they do not seem to form a phylogenetically homogeneous group that may have been derived from one or a few heterostylous ancestors. Therefore, heterostyly probably evolved multiple times through convergent selection pressures in the Rubiaceae (Anderson 1973).

Even in the genus *Hedyotis* (Rubiaceae-Hedyotideae), which includes 20 species of annual and perennial herbaceous plants found in the U.S., Canada, and Mexico, distyly likely evolved more than once. The genus is composed of two subgenera, *Houstonia* with six species, including *H. caerulea,* and *Chamisme* with 14 species. Of the six species in subgenus *Houstonia*, three are distylous perennials and three are homostylous annuals. In the subgenus *Chamisme*, nine are distylous perennials, two are distylous annuals, and three are homostylous annuals. There are no homostylous perennials in the genus.

Here the floral biology of several natural populations of *H. caerulea* in northeastern Ohio is examined in order to (1) evaluate the characteristics of the mating system found in this species in comparison with those of "true" distyly; and (2) determine

the relative stability of this species' mating system from an evolutionary perspective. If the species is distylous, its natural populations should have two distinct morphs in a one to one ratio. These morphs should display reciprocal herkogamy, as well as other floral polymorphisms. Intermorph mating should be strongly favored, at the expense of intramorph mating and selfing. In order to be considered evolutionarily stable, morphometric analysis of the floral characteristics should reveal genetic variation within the morphs, between the morphs, and across populations. Indications of gender specialization between the morphs would indicate a movement of the mating system towards dioecy. Indications of increased self-incompatibility between the morphs would indicate a movement towards selfing. However, if the mating system were characterized as truly distylous with no movement towards complete gender specialization or selfing, it would appear that natural selection is maintaining distyly within this species.

In order to test these predictions, ten populations of bluets were surveyed, the numbers of pin and thrum plants were counted to determine morph frequencies, and samples of each floral morph were collected. For each flower collected, anther height, stigma height, stigma length, and corolla tube widths were measured. These measurements were analyzed, and indices of reciprocal herkogamy were calculated and compared with other distylous species. Pollen diameter, papilla length, and dry weight were measured using flowers of both morphs collected during subsequent growing seasons. A controlled pollination was attempted in the field over three successive growing seasons to determine the level of self-compatibility of these flowers. The morphs were also compared by pollen count per anther and by count of seed produced per capsule from capsules that had matured in the field. Morph frequencies were

observed through season-long surveys in several populations. Floral development was studied through morphometric analysis and compared with the patterns found in similar studies of distylous species. In all morphometric studies, variation within morphs, between morphs, and across populations was analyzed.

CHAPTER II

QUANTITATIVE EVALUATION OF RECIPROCAL HERKOGAMY IN *HEDYOTIS CAERULEA*

Abstract

Reciprocal herkogamy (a reciprocal stigma-anther height polymorphism) has traditionally been necessary to characterize a flowering species as distylous. The placement of male and female reproductive organs within the two floral morphs, pins and thrums of distylous species, encourages legitimate (intermorph) pollen flow, resulting in an outcrossing advantage. Numerous species have often been described as distylous based solely on the observation of herkogamous flowers, without quantitative data establishing stigma-anther reciprocity in placement. Ten populations of *Hedyotis caerulea* located in the Cleveland Metroparks were surveyed and sampled during peak flowering. Equal numbers of pin and thrum flowers were randomly collected and measured for anther height, stigma height, and the difference between stigma height and anther height. Flower dry weights were measured in an independent sampling from three populations in the Cleveland Metroparks. Assessment of reciprocity was made using three different methods. Stigma and anther heights possessed bimodal distributions across

the ten populations surveyed with a slight overlap between morphs. The species clearly expressed two distinct morphs with little if any overlap in sizes of male and female reproductive characters between the two morphs. Significant variation among populations and significant population-by-morph type interactions suggest that the populations studied possess heritable differences upon which selection could act. Thus, the heights of the reproductive organs in this species have not yet become fixed. Thrum flowers, on average, are slightly heavier than pin flowers, a result that may be explained by the larger mass of the thrum's wider corolla tube. Two of the three measures used to assess reciprocity were inconsistent with precise symmetry. Further study of the plant's self-incompatibility system and its morph-frequency ratios are required in order to evaluate the biological significance of these deviations from precise reciprocity.

Introduction

The primary character used to define distyly is the presence of a reciprocal stigma-anther height polymorphism (Darwin 1877; Ganders 1979; Lloyd et al. 1990; Lloyd and Web 1992a). As the stigmas and anthers are spatially separated within a flower (herkogamy) the height of the pin stigmas within the flower matches that of the thrum anthers, and that of the pin anthers matches that of the thrum stigmas (Webb and Lloyd 1986; Lloyd and Webb 1992a). Called reciprocal herkogamy (Darwin 1877), this condition has traditionally been necessary and sufficient to characterize a flowering species as distylous (Richards, 1986; Webb and Lloyd, 1986). Darwin (1877) proposed that reciprocal herkogamy is adaptive within his Disassortative Pollen Flow Hypothesis. He predicted that pollen from the two morphs would be carried on different locations on pollinators' bodies. Thus, pollen from a pin flower would be positioned to be transferred

to the stigma of a thrum flower, and vice versa, resulting in greater proportion of legitimate pollen flow (pollen transfer in which the recipient and donor flowers are different morphs) than illegitimate pollen flow (in which the recipient and donor flowers are the same morph) compared to that expected from random placement of pollen. The resulting outcrossing advantage is thought to have driven the evolution and maintenance of distyly (Darwin, 1877; Lloyd and Webb, 1992a).

Since Darwin's pioneering work, numerous species often have been described as distylous based solely on the observation of herkogamous flowers, without quantitative data establishing stigma-anther reciprocity in placement. Here herkogamy in bluets (*Hedyotis caerulea*) has been evaluated, and to better assess potential variation in this mating system within the species, sampling was performed across 10 populations from natural areas in the region in order to present a proper assessment of reciprocity as put forth by Faivre and McDade (2001) and by Richards and Koptur (1993).

Materials and Methods

Ten populations of *Hedyotis caerulea* in northeast Ohio were surveyed and sampled during peak flowering in May and early June, 2005. All were located in the Cleveland Metroparks. Seven of the populations surveyed were in the Bedford Reservation, one in the North Chagrin Reservation, one in the Rocky River Reservation, and one in the Brecksville Reservation (see Table 2.1 and Fig. 2.1). These populations were chosen because they were of sufficient size to allow study over multiple growing seasons.

One flower from each individual plant was collected from each population. The flowers were then taken back to the lab, separated and counted by morph. Forty pin

flowers and forty thrum flowers from each population were selected at random by drawing them blindly from a pool of flowers of each morph in a Petri dish with the exception of Site 3, where the relatively small size of the population limited collection to only 20 pin individuals, although 40 thrum individuals were sampled. All flowers were preserved in 3% formalin for measurement of anther height, stigma height, and the difference between stigma height and anther height.

Anther height (AH) was measured from just above the ovary to the tip of the longest anther (see Figure 2.2); stigma height (SH) was measured from just above the ovary to the tip of the longer bifurcation of the stigma; difference between anther height and stigma height (D) was calculated as the absolute value of the difference AH – SH. Measurements were made in millimeters using an Olympus SZX12 dissecting microscope equipped with an ocular micrometer.

Flower dry weights were measured in an independent sampling during May, 2008. Twenty five pin and twenty five thrum flowers were collected from three bluet populations in the Cleveland Metroparks. The flowers were placed in individual weighing vials and heated in a drying oven for three days. The vials were weighed using a microbalance (Mettler AB54) first with the dried flower inside and then empty, and dry flower weights were calculated by subtraction.

For each population, variation among individuals of the same floral morph was assessed by the two characters and their difference described above and in Fig. 2.2 using one-way and two-way mixed-model ANOVA. Variation in dry weight between floral morphs was also assessed using a two-way ANOVA. All analyses were conducted in SAS (SAS, 1990). Assessment of reciprocity was made using three different methods:

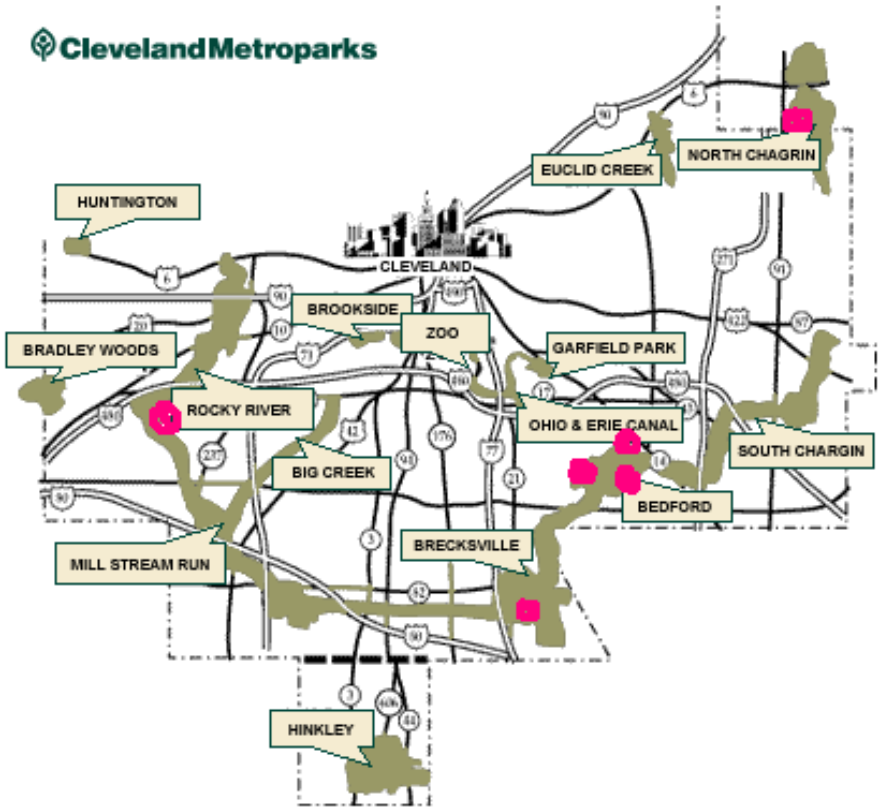

Figure 2.1 – A map of the Cleveland Metroparks indicating localities of populations studied.

Table 2.1: Locations and sizes of ten populations of *H. caerulea* surveyed showing variation in population sizes**.**

Locality No.	Site Location	N (No. Individuals)
1	**Buckeye Trail** Brecksville Reservation	**326**
2	**Emerald Circle Prairie** Bedford Reservation	**338**
3	**Girl's Scout Camp** Bedford Reservation	**68**
4	**Willis Picnic Area** Bedford Reservation	**543**
5	**Hemlock Creek** Bedford Reservation	**883**
6	**Hermit Hollow** Bedford Reservation	**254**
7	**Egbert (population 1)** Bedford Reservation	**89**
8	**Egbert (population 2)** Bedford Reservation	**200**
9	**Big Cedar Area** Rocky River Reservation	**825**
10	**Strawberry Hill Picnic Area** North Chagrin Reservation	**913**

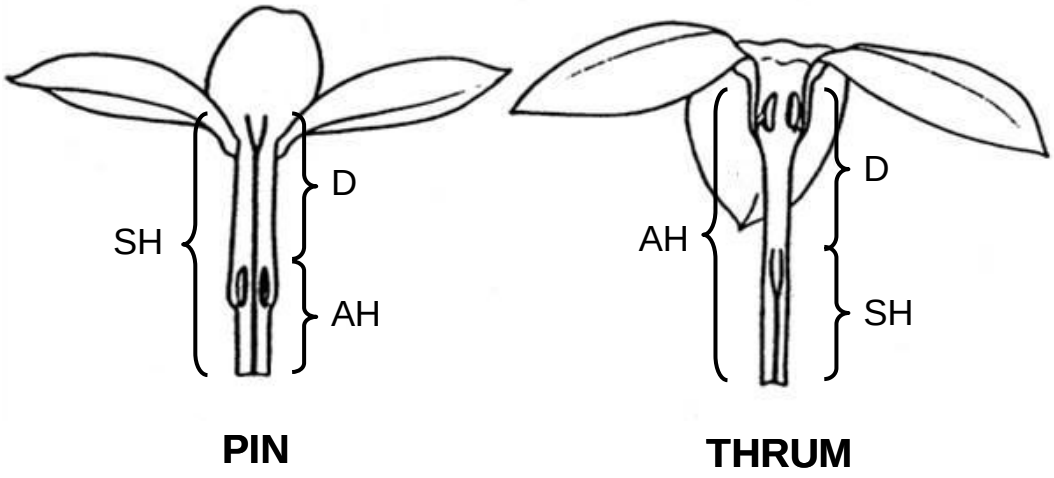

Figure 2.2 Floral measurements of *Hedyotis caerulea*. SH = stigma height; AH = anther height; D = the absolute value of the difference between the anther height and the stigma height.

(1) by comparison of the absolute value of anther height less stigma height (as in Faivre and McDade 2001); and (2) by measuring the relative reciprocity between organ levels (as in Richards and Koptur 1993) calculated for each organ level as (Anther Height – Reciprocal Stigma Height)/(Anther Height + Reciprocal Stigma Height).

Results

At a cursory glance the pin and thrum flowers looked similar enough that one could easily miss the features that distinguish them. Both morphs had diminutive, light blue flowers (occasionally white) with tubular corollas, a characteristic common among distylous flowers that is thought to confine pollen-bearing portions of the insect pollinators' bodies to predictable positions along the reproductive structures of the flowers. However, if one simply picked a flower and held it to eye level, one could see a dark spot within the corolla tube, which was actually the presence of four anthers within the corolla tube. In pin flowers, the dark spot appeared at the base of the corolla tube, just above the ovary. In thrum flowers, the spot was visible higher in the corolla tube just where the tube splits into petal-like structures. Since the anther collective appeared nearly as wide as the corolla tube itself, the width of the tube varied with the position of the anthers. Thus, in pins, the corolla tube was relatively uniform in width from the base to the petals. In thrums, the tube was narrow at its base and flared wide below the petals in a shape somewhat resembling a trumpet. These observations suggested that pin and thrum flowers differ in ways that are not obvious by external inspection of the flower alone.

Quantitatively, stigma and anther heights expressed bimodal distributions across the ten populations surveyed (see Figure 2.3). However, the stigma-anther height

differences showed a continuous distribution with a nearly equivalent number of pins and thrums in each height class with no significant variation among populations (see Figure 2.6). When comparing the two morphs (see Figures 2.4 and 2.5, the mean stigma and anther heights differed significantly (stigma height, $F_{1,9} = 1310$, $P < 0.0001$; anther height $F_{1.9} = 1836$, $P < 0.0001$). In the ANOVA for stigma height, between-morph variation accounted for 73% of the total variation; for anther height, between-morph variation accounted for 90% of the total variation. However, locality had a significant effect on stigma height in both pins ($F_{9,390} = 8.29$, $P < 0.0001$) and thrums ($F_{9,370} = 5.53$, $P < 0.0001$) and in anther height in thrums ($F_{9,370} = 6.47$, $P < 0.0001$). Locality did not have a significant effect on anther height in pins ($F_{9,390} = 2.43$). There was a statistically significant interaction between morph and locality on anther heights ($F_{9,760} = 4.27$, $P < 0.0001$) due to variation of flower size at the different sites as quantified by flower dry weights. The mean dry weight for a pin flower was 1.183 ± 0.59 mg, and for a thrum flower 1.443 ± 0.071 mg. While the difference in dry weight between morphs was significant ($F_{1,144} = 6.79$, $P < 0.0101$), locality effects accounted for a greater amount of the variation among individuals across the three populations sampled ($F_{2,144} = 16.0$, $P < 0.0001$). There was no interaction effect between morph and locality.

Across the ten populations, the mean pin stigma height was 5.95 ± 0.097 mm (CV = 0.33) with a span of 0.93 mm from smallest to largest population mean, while the mean thrum stigma height was 3.79 ± 0.065 mm (CV = 0.33) with a span of 0.76 mm (see Figure 2.4). The mean pin anther height across these ten populations was 3.16 ± 0.025 (CV = 0.29) with a span of 0.25 mm from smallest to largest population mean, while the

mean thrum anther height was 6.62 ± 0.099 (CV = 0.33) with a span of 1.06 mm (see Figure 2.5).

Reciprocity of organ placement was assessed three ways. First, the mean heights of the two long organs were compared (see Figure 2.4 and 2.5), and then the mean heights of the two short organs were compared. The mean height of the pin stigmas (5.95 mm) and mean height of the thrum anthers (6.62 mm) differed significantly ($P < 0.0001$). Similarly, the mean height of the pin anthers (3.11 mm) and the thrum stigmas (3.79 mm) also differed significantly ($P < 0.0001$). Secondly, the difference between stigma and anther heights were analyzed between morphs (see Figure 2.6), and this variation was minor (F = 0.53, n.s.). Instead, a significant locality effect was shown in both morphs ($F_{9,760} = 8.61$, $P < 0.0001$), as well as an interaction between locality and morph ($F_{9,760} = 7.68$, $P < 0.0001$) and these differences related more to size variation among flowers from these populations. Third, relative reciprocity indices for long and short organ levels for all ten populations gave a mean ratio for the long organs of -0.091 and that for the short organs of 0.053 (see Figure 2.7).

Discussion

In spite of the uniform external appearance of *Hedyotis caerulea* flowers, variation in floral morphology is pronounced both within and among these populations. The species clearly maintains two distinct morphs with little, if any, overlap in sizes of male and female reproductive characters. The two morphs differ in regard to the heights of their stigmas and anthers, which defines the flowers that *H. caerulea* displays as distylous, meets the first of three criteria needed to qualify their mating system as distylous. While the majority of the variation found within and between populations was

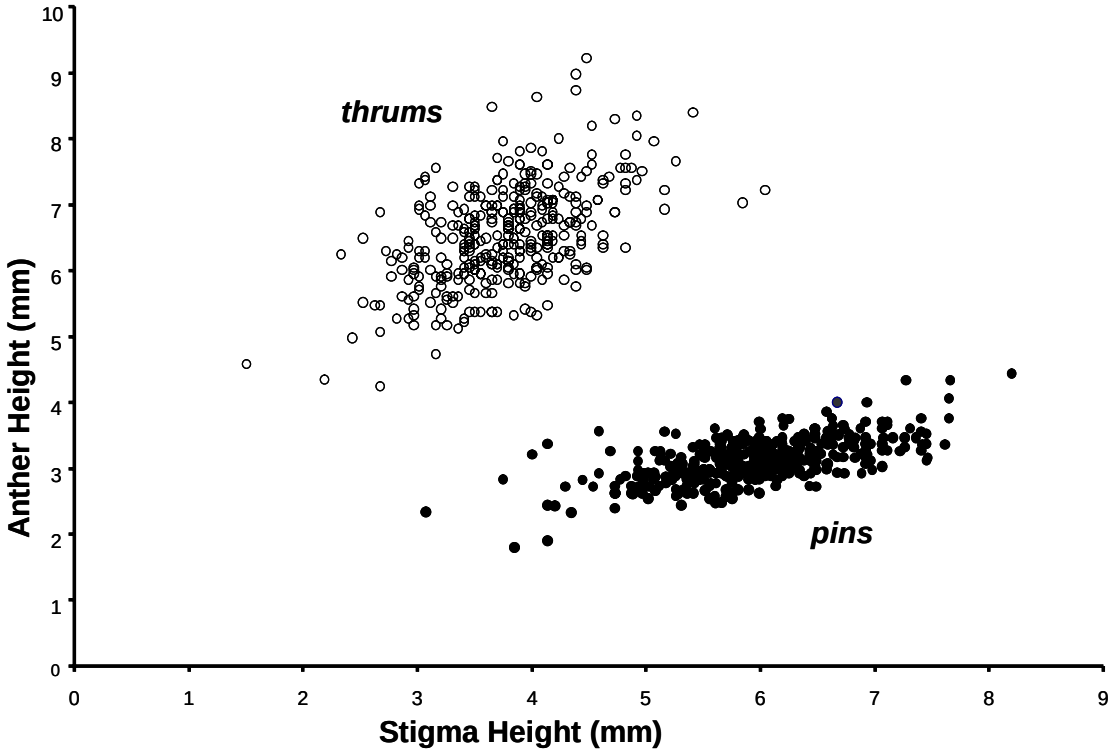

Figure 2.3 Scatterplot representing the anther and stigma heights of flowers measured. Each point represents an individual flower with its placement along the X axis indicating its stigma height (in mm) and its placement along the y-axis indicating its anther height (in mm).

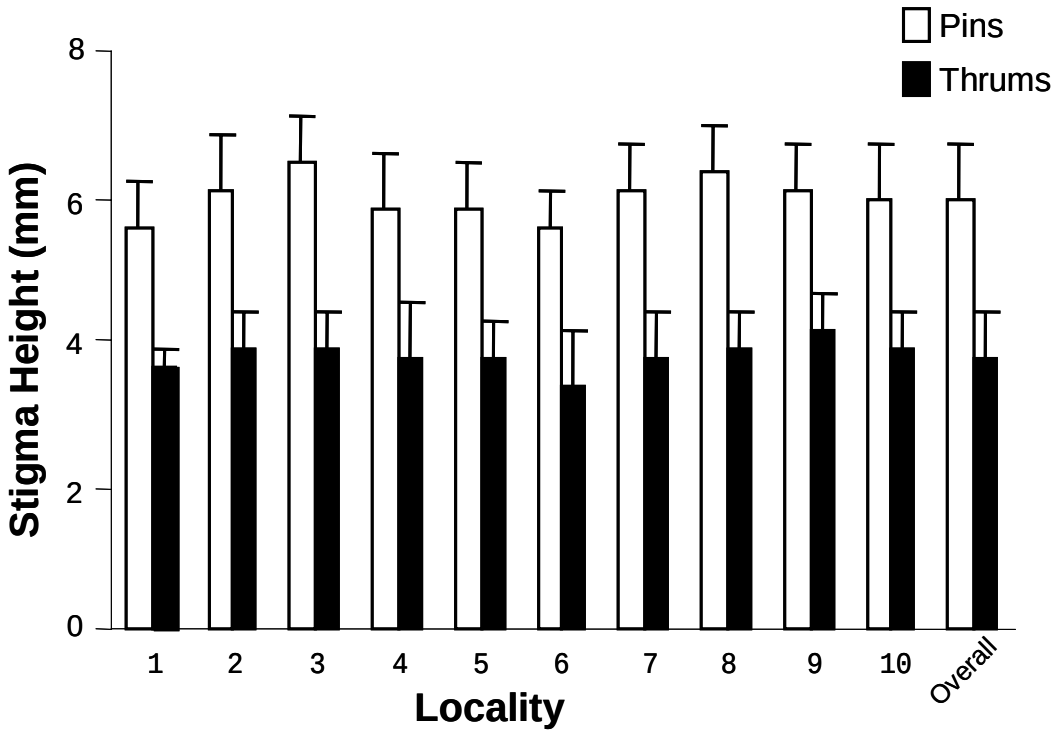

Figure 2.4 – Mean stigma height (in mm) by morph across ten populations of *H. caerulea.* The error bars represent standard errors.

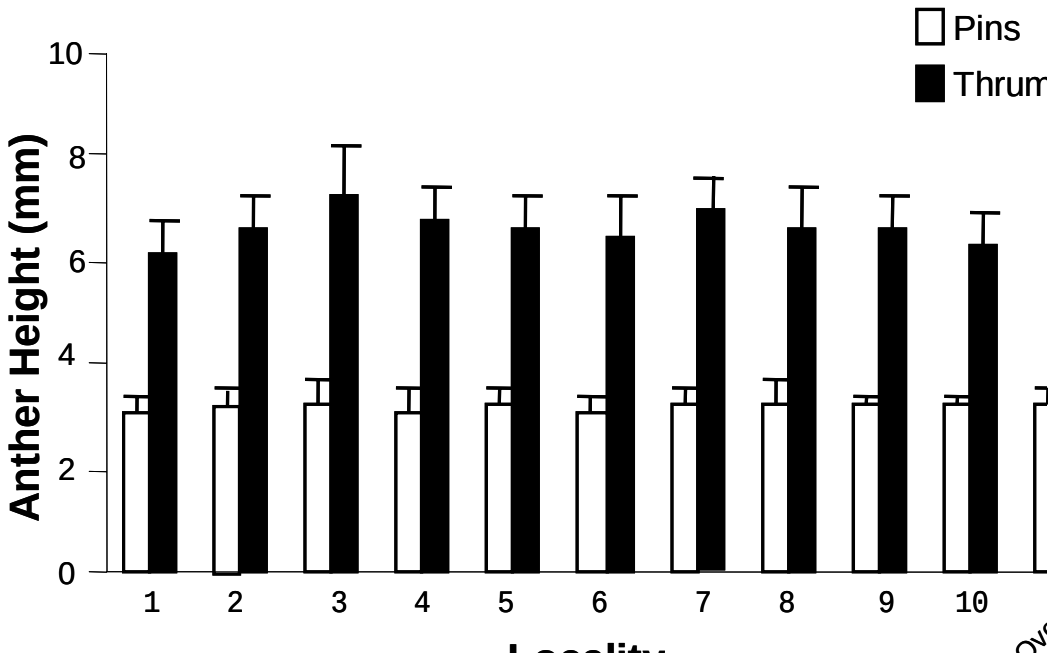

Figure 2.5 – Mean anther height (in mm) by morph across ten populations of *H. caerulea.* The error bars represent standard errors.

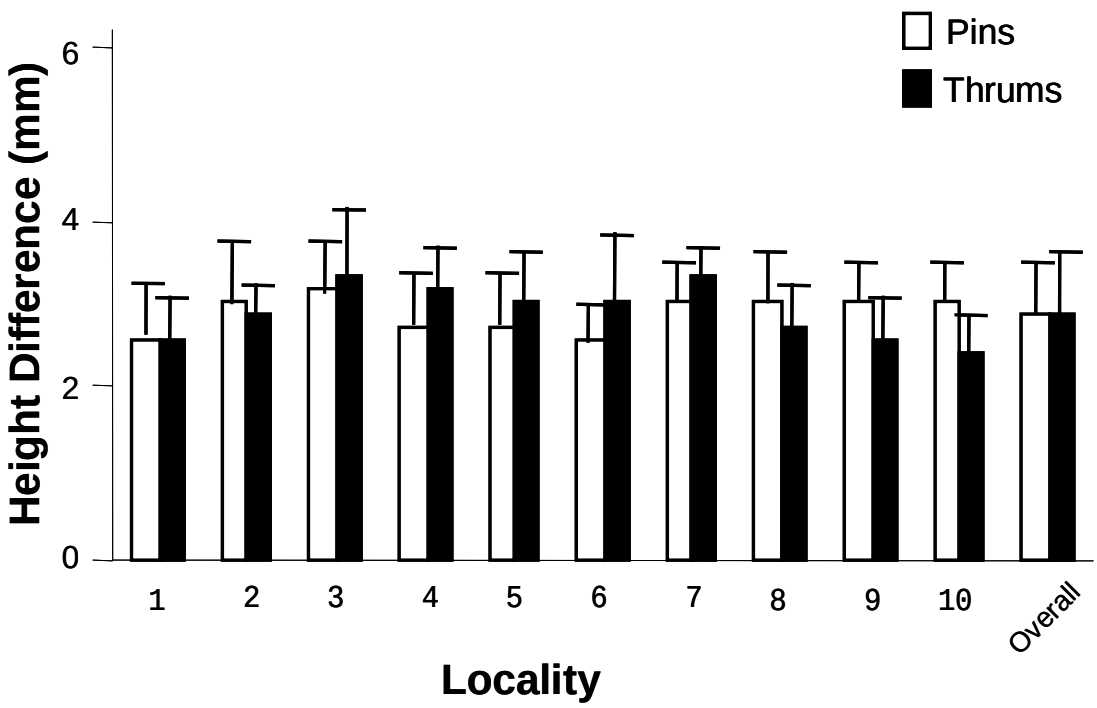

Figure 2.6 – Mean absolute value of difference between anther height and stigma height (in mm) by morph across ten populations of *H. caerulea.* The error bars represent standard errors.

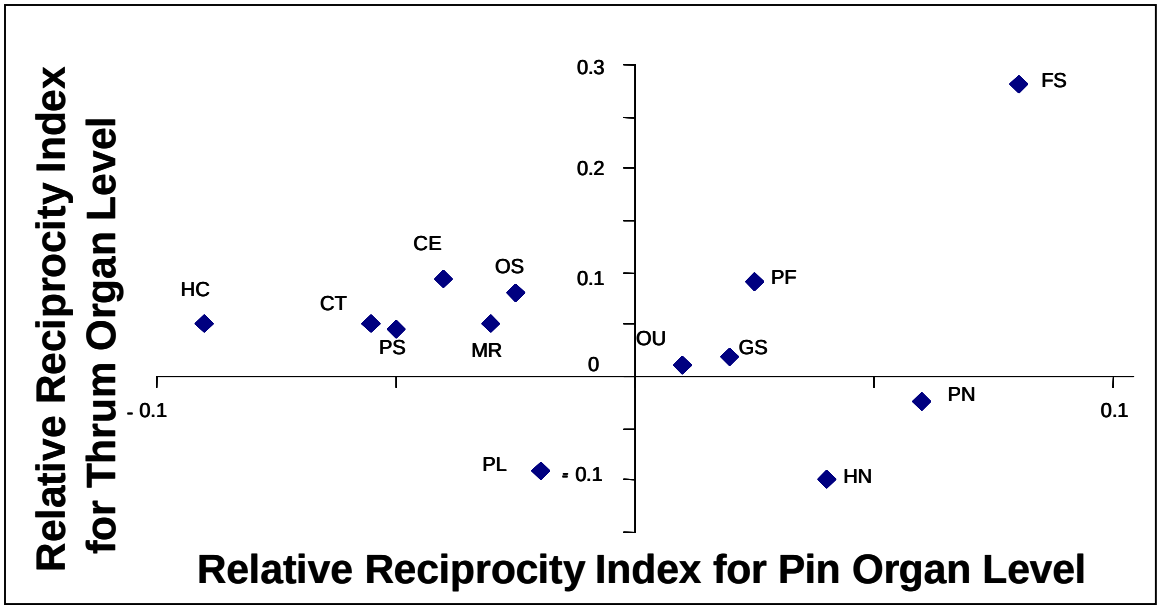

Figure 2.7 Relative reciprocity ratio for the pin organ level vs. the short organ level for distylous species in the Rubiaceae, including new data from *H. caerulea*. Abbreviations for species are adjacent to each point on the graph. Abbreviations are: HC = *H. caerulea*; CE = *Cephaelis elata*; CT = *Cephaelis tomentosa*; FS = *Faramea suerrensis*; GS = *Guettarda scabra*; HN = *Hedyotis nigricans*; MR = *Mitchella repens*; OU = *Oldenlandia umbellata*; OS = *Oldenlandia scopulorum*; PF = *Palicourea fendleri*; PL = *Pentas lanceolata*; PN = *Psychotria nervosa*; PS = *Psychotria suerrensis*. Adapted from Richards and Koptur 1993.

due to intermorph differences, these flowers also showed a also great deal of intramorph variation, as well as a marked degree of interpopulation variation in some instances. While environmental causes cannot be entirely dismissed without further study, significant variation among populations and significant population-by-morph type interactions tentatively suggest that the populations studied possess heritable differences upon which selection could act. Thus, the heights of the reproductive organs in this species have not yet become fixed.

Several authors have stated that reciprocal herkogamy (i.e., having exact reciprocity of placement between stigmas and anthers between floral morphs) is the minimum criterion for a species to be considered distylous. From a theoretical perspective, precise reciprocity would allow intermorph pollination in a distylous population to be most efficient and expresses a perfectly symmetrical exchange of pollen; i.e. functionally equivalent amounts of pollen would be carried from one morph to the other. Pollen would be transferred with equal efficiency when attached to pollinators at one of two positions on the pollinator's body – the position at which it carries pollen from long anthers and pollen from short anthers. The pollinator would then be prepared to pollinate stigmas that are both long and short through legitimate pollination.

It is doubtful, however, that a mating system such at this would actually require precise symmetry, as small differences, even if statistically significant, would not necessarily be biologically significant. Such a deviation from the ideal was evidenced in *H. caerulea* by the difference in the size of the two morphs. Thrum flowers, on average, are slightly heavier than pin flowers, a result that may be explained by the larger mass of the thrum's wider corolla tube. Larger flower size could underlie a greater energetic

investment in thrum flowers, but, in spite of the asymmetry created by the differences in weight, there was no evidence to indicate differential fitness of the two morphs. While Ganders (1979) found that, for flowers of *Lithospermum californicum* (Boraginaceae), differences of as little as 2 mm in stigma and anther height in pin flowers significantly affected the degree of legitimate pollination, the corresponding differences in flowers of *H. caerulea* were much smaller.

Also, two of the three measures used to assess reciprocity were out of line with symmetry. In order to determine the degree to which distylous plants actually present the ‘‘typical’’ heterostylous syndrome, the absolute value of the difference between anther and stigma heights should be identical between floral morphs (Faivre and McDade 2001). The results presented here show no significant difference in these values between pin and thrum flowers. However, a more direct comparison of the mean heights of the two long organs and then the two short organs revealed a statistically significant difference between values that would be equal if strong reciprocity for sex-organ position existed between the two morphs. Third, the relative reciprocity index (R) of the short organs is within the range observed in other Rubiaceae (Richards and Koptur 1993; Pailler and Thompson 1997), although the R value for the long organs suggests their heights are less strongly reciprocal. In order to evaluate the biological significance of these deviations from precise reciprocity, further studies of the plant’s self-incompatibility system and its morph-frequency ratios are required.

CHAPTER III

VARIATION IN ANCILLARY FLORAL CHARACTERS AND THEIR RELATIONSHIP TO THE EVOLUTION OF HETEROSTYLY

Abstract

In addition to reciprocal herkogamy and a self-incompatibility system, the "heterostylous" syndrome usually includes several, less prominent "ancillary" polymorphisms involving such characteristics as pollen production, pollen size, size of the stigmatic papillae, and corolla diameter. These ancillary floral polymorphisms have been the least investigated aspects of distyly, and thus their functional significance and evolution in distylous species remain obscure. Stigma length, width of corolla tube base, and width of corolla tube top of the flowers were measured on the same flowers of *Hedyotis caerulea* collected and measured previously for anther height, stigma height, and difference between anther height. Papilla length and pollen size were measured on separate collections of flowers. Width of the corolla tube, measured at its base and its mouth, showed significant differences between morphs. Pins were wider at the base and thrums were wider at the top. No significant differences between pin and thrum flowers were found in stigma lengths. Pin papillae were significantly longer than thrum papillae,

while pin pollen were smaller than thrum pollen. Analysis of correlations among the characters measured produced weak correlations across the matrix. In pin flowers, ancillary characters of the stigma showed stronger correlations than those of the anthers. In thrum flowers, these ancillary characters of the anthers showed larger correlations than those of the stigma.

Among each of these trait sets that are related to fitness – corolla tube shape, stigma morphology, and pollen size – variation across the populations sampled was almost always significant and generally considerable. Population differences are commonly considered to reflect genetic differences and the possibility for the traits to change over time. The ancillary traits discussed here likely shift evolutionarily in response to subtle changes in as yet unidentified selection pressures.

Introduction

In addition to reciprocal herkogamy and a sporophytically controlled, diallelic self-incompatibility system, the "heterostylous" syndrome usually includes several, less prominent "ancillary" polymorphisms involving such characteristics as pollen size, pollen production, size of the stigmatic surface, corolla diameter, and style pubescence (Ganders 1979; Dulberger 1992; Richards 1997). Of the characteristics that define heterostyly, ancillary polymorphisms have been least investigated and their functional significance and evolution remain obscure. In published descriptions of distylous species (reviewed in Ganders 1979 and Dulberger 1992), these ancillary polymorphisms have traditionally been scored as characters without analysis, probably because the details of the polymorphic characters vary between genera and families, and there are numerous exceptions across taxa to any generalization one might use to describe the dimorphism.

The most common ancillary polymorphism shared by most distylous taxa is pollen size. In general, pollen grains from thrum flowers are larger than those from pin flowers (e.g., *Primula vulgaris*: Ornduff 1979; *Fagopyrum esculentum*: Dulberger 1992; *Cratoxylum formosum*: Lewis 1982). However, there are a significant number of exceptions to this generalization. In some distylous species, the pin flowers have larger pollen grains (e.g. *Linum flavum*: Punt & den Breejen 1981; *Limoniastrum feei*: Weber 1981), and others reportedly lack size dimorphism (e.g., *Amsinckia spectabilis* var. *spectabilis*: Ray & Chisaki 1957; *Linum pubescens*: Dulberger 1973). In most species examined, morphs also differ in pollen production, with pin flowers generally producing more pollen than those of thrum flowers (Ganders 1979).

A number of characters associated with stigmas have also been described as polymorphic. The receptive surface of the pin morph is typically larger than that of the thrum (*Jepsonia parryi*: Ornduff 1970). In contrast, however, thrum stigmas are larger than pin stigmas in *Amsinckia grandiflora* (Ornduff 1976) and *Primula malacoides* (Pandey and Troughton 1974). In *Rudgea jasminoides*, thrum stigmas are long, narrow, and curled, whereas pin stigmas are short and flat (Baker 1956). The most frequently reported polymorphism of stigmas is that papillae in pins are larger than those in thrums (Vuilleumier 1967; Dulberger 1974). However, actual measurements of papilla size are documented in relatively few plants (e.g., *Lythrum junceum*: Dulberger 1970; *L. curtisii*: Ornduff 1978; *Pulmonaria obscura*: Oleson 1979).

While the nature of these polymorphism may be complex, researchers (Mather & de Winton 1941; Dulberger 1975) have suggested that the associations between these traits may function in an integrated fashion and may have evolved together to promote

cross-fertilization (Darwin 1877; Lloyd & Webb 1992b). Thus, quantitative analysis of the ancillary traits in heterostylous species may shed light on the functional significance of these floral characters and will improve the understanding of the nature and evolution of heterostyly.

Materials and Methods

Stigma length (SL), width of corolla tube base (CB), and width of corolla tube top (CT) (see Figure 3.1) were measured on the same flowers collected and measured previously and scored for anther height, stigma height, and difference between anther height (see Table 2.1 and Fig. 2.1 for localities of the populations sampled). Measurements were made in millimeters using an Olympus SZX12 dissecting microscope equipped with an ocular micrometer. Papilla length and pollen size were measured on separate collections of flowers.

Papilla length was measured in an independent sampling of five of these populations (locality # 1 in the Brecksville from Table 2.1, locality # 10 in the North Chagrin, localities # 2 and # 7 in Bedford, and locality # 9 in Rocky River) in May of 2007. Fifteen pin flowers and 15 thrum flowers were collected from each site, and were preserved in 70% ethanol. The lengths of four papillae on each stigma were measured in micrometers using a microscope (Leitz Dialux EB20) equipped with an ocular micrometer. The most clearly visible papilla in the best-focused field of view was measured on each side of the stigma's bifurcation. For each population, variation among individuals of the same floral morph was assessed by the six characters described above using one-way and two-way mixed-model ANOVA. Correlations among stigma height, stigma length, anther height, distance between anther and stigma height, width of corolla

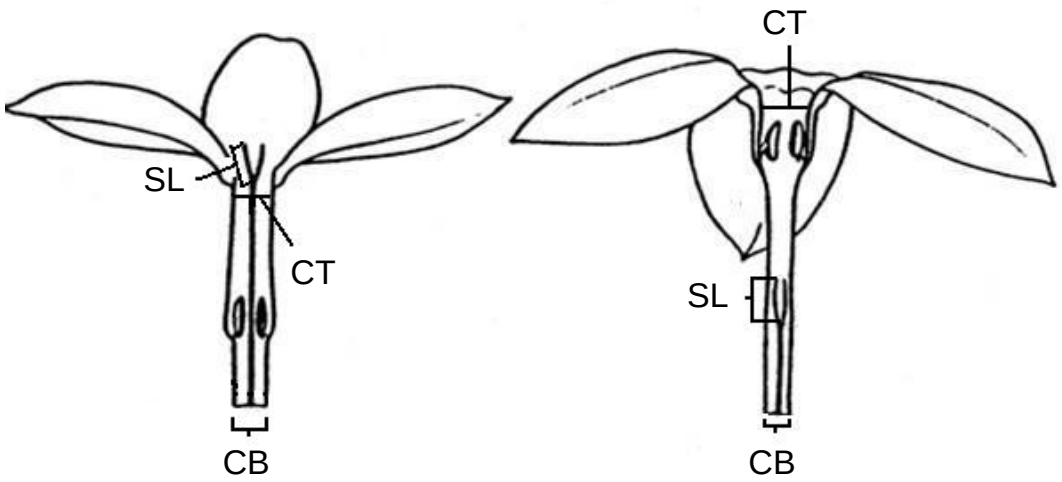

Figure 3.1 Floral measurements of *Hedyotis caerulea*. CT = width of the corolla tube at the top; CB = width of the corolla tube at the bottom; SL = stigma length.

tube tops, and width of corolla tube base were assessed. All analyses were conducted in SAS 9.1 (SAS 2002).

Results

The most obvious morphological difference between the two morphs was the shape of the corolla tube (see Figure 3.1). In thrums, the anthers were located at the mouth of the tube, and the width of the corolla tube was correspondingly greater at the mouth to make room for the anthers. Below the position of the anthers, the corolla tube narrowed as it approached the ovary, making the base of the tube much narrower than the top. In pins, the anthers were located at the base of the corolla tube, near the ovary. To accommodate this position of the anthers, the base of the corolla tube was much wider at the base than it was in the thrum flowers, and there was little difference between the widths at the top and the base of the pin corolla tube. These differences were verified by morphometric analysis. Corolla tubes differed in width between morphs (Figures 3.2 and 3.3), and were larger at the base in pins ($F_{1,9} = 1277$, $P < 0.0001$) but larger in thrums at the top of the tubes ($F_{1,9} = 598$, $P < 0.0001$). When the corolla tubes were measured at the base, the mean pin width was 1.14 ± 0.01 mm (CV = 0.15) with a span of 0.27 mm from the smallest to largest population mean, while the mean thrum width was 0.80 ± 0.01 mm (CV = 0.15) with a span of 0.09 mm across populations. When the corolla tubes were measured at the top, the mean pin width was 1.47 ± 0.01 mm (CV = 0.12) with a span of 0.21 mm from the smallest to largest population mean, while the mean thrum width was 1.80 ± 0.01 mm (CV = 0.11) with a span of 0.14 mm among populations. Morph, therefore, accounted for the majority of the variation in width of corolla tube, but variation among localities was significant for width of the tube base

($F_{9,760} = 14.12$, $P < 0.0001$). The locality effect was significant in both pins ($F_{9,390} = 16.72$, $P < 0.0001$) and thrums ($F_{9,370} = 2.77$, $P < 0.001$). An interaction between morph and locality ($F_{9,760} = 6.17$, $P < 0.0001$) was significant, as well. Similarly, there was a significant locality effect among populations for width of tube top ($F_{1,9} = 7.07$, $P < 0.0001$), although no consistent geographic trend was suggested (see Table 3.1). This effect appeared in both pins ($F_{9,390} = 5.98$, $P < 0.0001$) and thrums $F_{9,370} = 3.46$, ($P < 0.001$). However, there was no significant interaction between morph and locality ($F_{1,9} = 2.22$).

Unlike the corolla tubes, the stigmas of the pins and thrums did not appear to differ appreciably, an observation that also was verified through morphometry. Across the ten populations, the mean pin stigma length was 1.36 ± 0.02 mm (CV = 0.24) with a span of 0.34 mm from the smallest to largest population mean, and the mean thrum stigma length was 1.37 ± 0.02 mm (CV = 0.22) with a span of 0.45 mm. Therefore, these values overlapped to a great degree (see Figure 3.4). When comparing the stigma lengths measured, morph did not contribute significantly to the variation ($F_{1,9} = 0.53$); rather, locality ($F_{9,760} = 8.61$, $P < 0.0001$) and an interaction between morph and locality ($F_{9,760} = 7.86$, $P < 0.0001$) accounted for a significant proportion (16%) of the variation. One-way ANOVAs suggested that locality had a significant effect in both pins ($F_{9,390} = 7.37$, $P < 0.0001$) and thrums ($F_{9,370} = 8.90$, $P < 0.0001$), although no consistent geographic trend was suggested (see Tables 3.3 and 3.4).

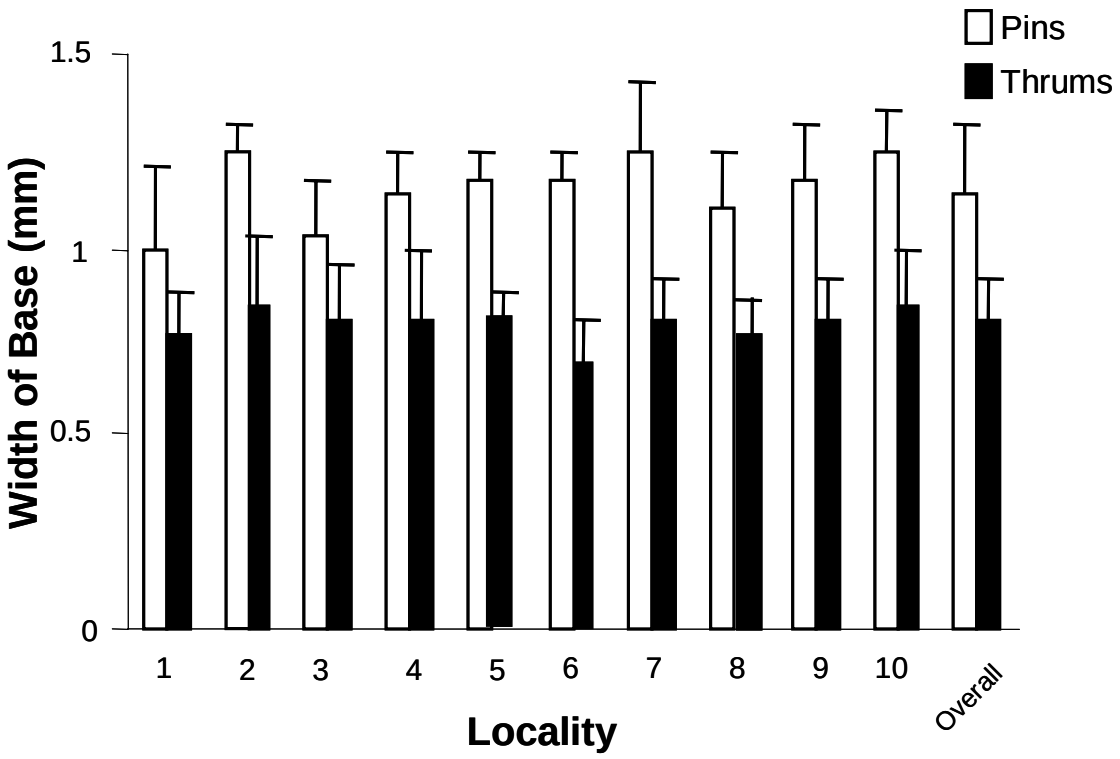

Figure 3.2 Mean width (in millimeters) of corolla tube (at base) across ten populations of *H. caerulea.* Error bars represent standard errors.

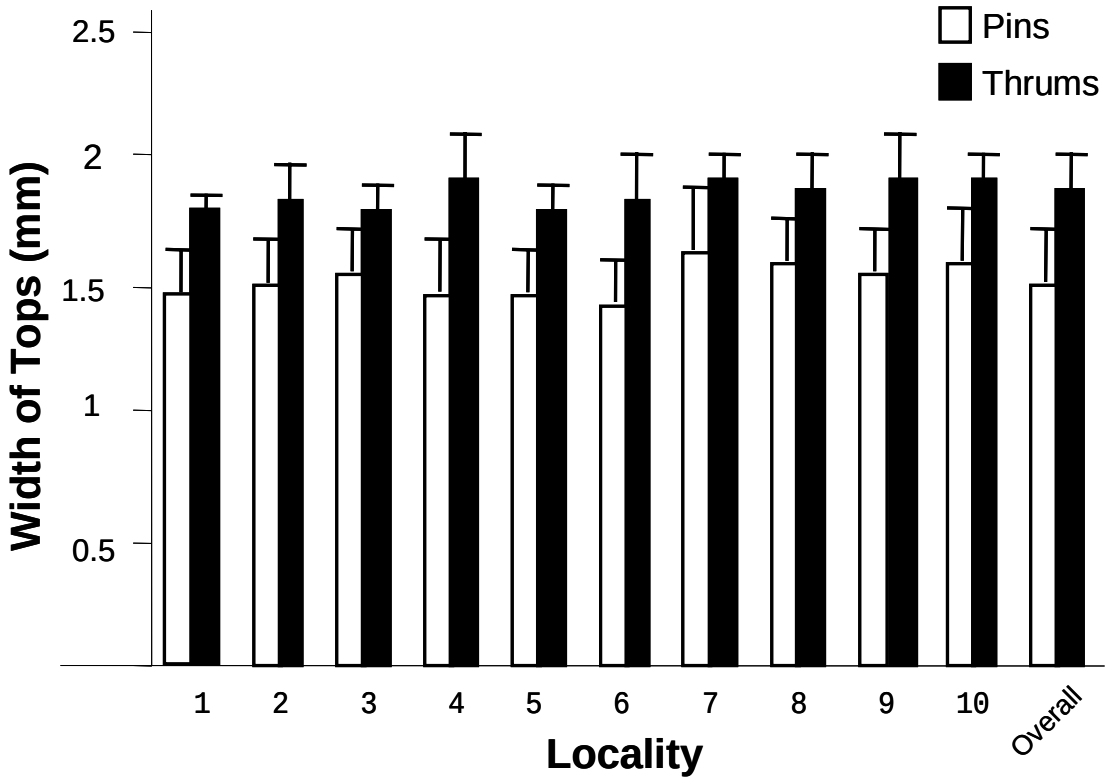

Figure 3.3 Mean width of corolla tubes (at top) across ten populations of *H. caerulea.* Error bars represent standard errors.

Table 3.1 Tukey groupings for width of corolla tops in pins. Means with the same letter are not significantly different.

Locality	Mean (mm)	N	Tukey Groupings			
7	1.59	40			A	
8	1.55	40	B		A	
10	1.53	40	B		A	C
9	1.48	40	B	D	A	C
3	1.48	40	B	D	A	C
2	1.44	40	B	D		C
1	1.43	40	B	D		C
5	1.42	40		D		C
4	1.41	40		D		C
6	1.38	40		D		

Table 3.2 Tukey groupings for width of corolla tops in thrums. Means with the same letter are not significantly different.

Locality	Mean (mm)	N	Tukey Groupings	
7	1.86	40	A	
9	1.86	40	A	
10	1.85	40	A	B
4	1.85	40	A	B
8	1.83	40	A	B
6	1.79	20	A	B
2	1.78	40	A	B
1	1.73	40	A	B
3	1.72	20	A	B
5	1.72	40		B

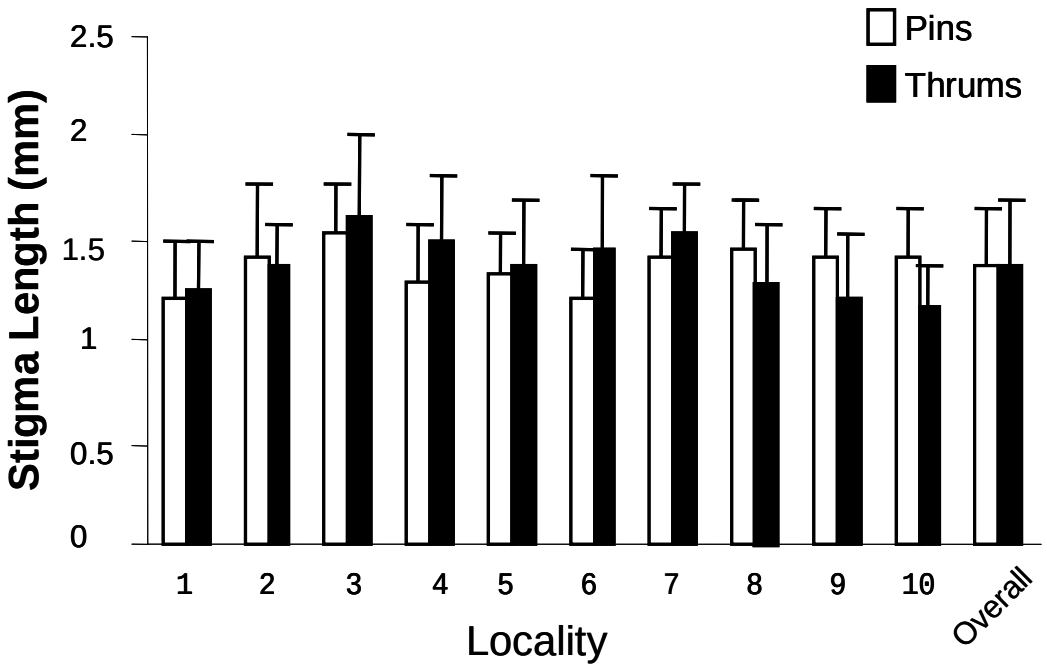

Figure 3.4 Mean stigma length (in millimeters) across ten populations of *H. caerulea*. Error bars represent standard errors.

Table 3.3 Tukey groupings for stigma length in pins. Means with the same letter are not significantly different.

Locality	Mean (mm)	N	Tukey Groupings	
3	1.53	40		A
8	1.46	40	B	A
7	1.43	40	B	A
2	1.42	40	B	A
9	1.42	40	B	A
10	1.41	40	B	A
5	1.32	40	B	C
4	1.28	40	B	C
1	1.20	40		C
6	1.19	40		C

Table 3.4 Tukey groupings for stigma length in thrums. Means with the same letter are not significantly different.

Locality	Mean (mm)	N	Tukey Groupings			
3	1.60	20	A			
7	1.54	40	A			
4	1.49	40	A	B		
6	1.46	40	A	B		
5	1.39	40	A	B	C	
2	1.37	40	A	B	C	
8	1.31	40		B	C	D
1	1.25	40			C	D
9	1.22	40			C	D
10	1.15	40				D

Analysis of correlations among stigma height, anther height, the difference among stigma height and anther height, stigma length, corolla tube width at the top, and corolla tube width at the base largely showed weak correlations across the matrix (see Table 3.5). In pin flowers, there was a strong correlation ($P < 0.0001$) between the corolla tube width at the top and at the base (reflecting the shape of the pin corolla tube). Features of the stigma showed stronger correlations than features of the anthers. Corolla tube width at the top also corresponded strongly ($P < 0.0001$) with stigma height, stigma length, and the difference between stigma height and anther height. Stigma length also correlated ($P < 0.0001$) strongly with stigma height and distance between stigma height and anther height.

In thrum flowers, the corolla tube width at the base and corolla tube width at the top also showed a strong correlation ($P < 0.0001$), but features of the anthers showed greater correlations than features of the stigma. Anther height correlated strongly ($P < 0.0001$) with stigma length, stigma height, corolla tube width at the top, and difference between stigma height and anther height. However, as in pins, stigma height and stigma length also correlated strongly ($P < 0.0001$) in thrums.

The structure of the stigma appeared to be a simple extension of the style after the tissue on the distal end split into two forks. Papillae projected out from the surface of the stigmatic bifurcation with no apparent difference between the two morphs, although measurement revealed mean length of the papillae differed significantly between morphs ($F_{1,4} = 267$, $P < 0.0001$; see Figure 3.5). Across all five populations, papilla length was longer in pins (0.047 ± 0.001 mm, CV = 0.26 with a span of 0.011 mm from smallest to largest population mean) than in thrums (0.029 ± 0.0004 mm, CV = 0.28 with a span of

Table 3.5 Correlation Matrix for width of corolla tube top (CT), width of corolla tube base (CB), height of stigma (SH), distance between stigma and anther (DSA), stigma length (SL), and height of anther (AH). All measurements were taken in millimeters. Values for correlation of pin measurements (N = 400) are on the top portion of the matrix, while values for correlation in thrums (N = 380) are on the base.

	CT	**CB**	**SH**	**DSA**	**SL**	**AH**
CT		0.38 <.0001	0.24 <.0001	0.19 0.0001	0.23 <.0001	0.08 0.13
CB	0.41 <.0001		0.14 .0056	0.18 0.0003	0.13 0.011	0.06 0.21
SH	0.19 0.0002	0.17 0.0007		0.89 <.0001	0.51 .0001	0.18 0.0002
DSA	0.093 0.070	-0.04 0.42	-0.27 <. 0001		0.53 <.0001	0.09 0.063
SL	0.084 0.10	0.07 0.17	0.50 <. 0001	-0.17 0.0009		0.03 0.61
AH	0.23 <.0001	0.10 0.056	0.53 <. 0001	0.67 <. 0001	0.24 <. 0001	

0.005 mm, Figure 3.5). A two-way mixed-model ANOVA showed a significant effect between morphs ($F_{1,4} = 267$, $P < 0.0001$), a significant interaction between individual flower sampled within locality ($F_{70,450} = 3.79$, $P < 0.0001$), a significant interaction between locality and morph ($F_{4,70} = 6.07$, $P < 0.0001$), and a significant interaction between morph and individual by locality ($F_{70,450} = 3.40$, $P < 0.0001$). However, locality as a factor by itself made highly significant contributions to the variation when the morphs were analyzed separately ($F_{4,225} = 4.08$, $P < 0.0001$ in pins and $F_{4,225} = 7.38$, $P < 0.0001$ in thrums), but insignificantly when morphs were combined in the analysis ($F_{4,70} = 1.27$, n.s.). An interaction between individual flower and locality was significant in both morphs (pins, $F_{70,225} = 3.77$, $P < 0.0001$ and thrums, $F_{70,225} = 3.23$, $P < 0.0001$).

A difference in the size of the pollen grains was also obvious, however, when pin pollen and thrum pollen were observed side by side under a microscope ($F_{1,5} = 98.3$, $P < 0.0001$). The mean pin diameter was 24.78 ± 0.16 μm (CV = 0.16), with a 1.56μm span from smallest to largest population mean across six populations (see Figure 3.6). The mean thrum diameter was 29.72 ± 0.20 μm (CV = 0.12), with a 3.6μm span. One-way ANOVAs showed no significant locality effect in pins ($F_{5,270} = 3.20$), but there was a significant interaction between individual flower sampled and locality ($F_{24,270} = 5.28$, $P < 0.0001$). In thrums, locality was significant ($F_{5,270} = 14.86$), and there was also a significant interaction between individual and locality ($F_{24,270} = 8.57$, $P < 0.0001$).

Discussion

The enclosure of the flower's sex organs within a tube beneath the petals is characteristic of distylous species (Ganders 1979). Since the pollen is held either above or below the stigma inside the tube, the space in which a pollinator can move is strictly

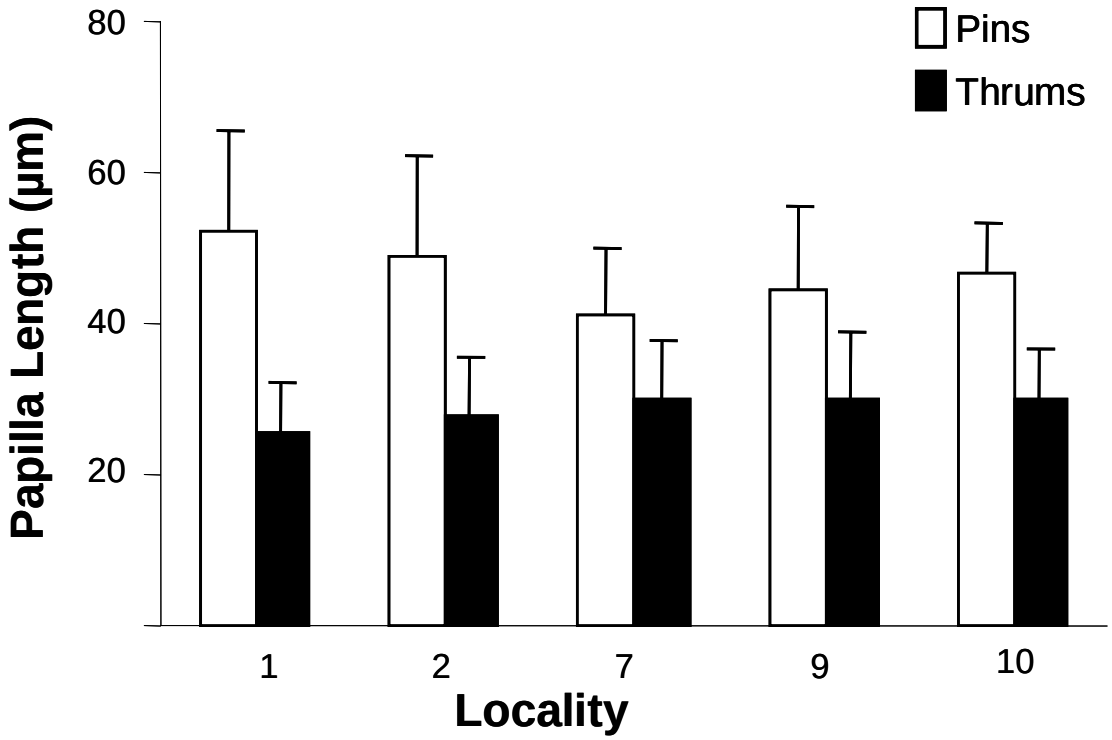

Figure 3.5 Mean papilla length (in microns) across five populations of *H. caerulea.* Error bars represent standard errors.

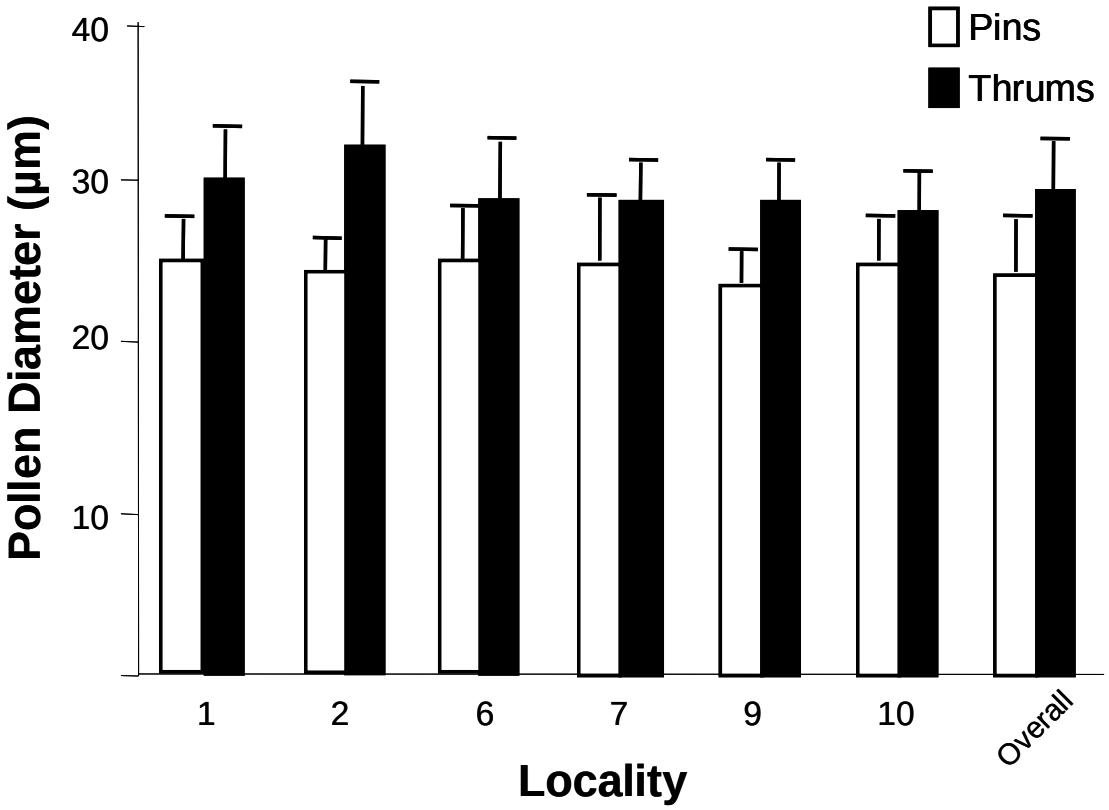

Figure 3.6 Mean pollen diameter (in microns) across six populations of *H. caerulea* Error bars represent standard errors.

limited, presumably facilitating precise legitimate pollen deposition. In *H. caerulea*, the corolla tube width of each morph is only slightly larger than the collective width of the anthers, which creates a very narrow passageway for pollinators to explore, encouraging contact with both floral sex organs. The differential placement of the anthers within the corolla tube causes the morphs to differ in shape. Width of the corolla tube, measured at its base and its mouth, showed significant correlation upon analysis and helped create the distinct shapes of the two flower morphs. A pin corolla tube resembles a water glass, with a base just wide enough to hold the anthers, and then becomes slightly (1.3 times) wider at the mouth. In contrast, the corolla tube in a thrum flower is more than two times wider at the mouth than at the base caused by a bulge created by the anthers positioned there. The tube narrows dramatically just below this bulge and continues to the ovary, creating a shape that resembles a fluted champagne glass with the thrum stigma located just above the ovary enclosed in the narrowest portion of the corolla tube. Thus, pins are wider at the base and thrums are wider at the top. Since pin anthers are located near the base of the tube, corolla tube width at the top correlated most weakly with anther height and correlated strongly with stigma height and difference between stigma height and anther height. Conversely, thrum corolla tube width at the top (where anthers are positioned) correlated most strongly with anther height.

The morphology of the stigma has functional significance as an important component of receptive surface area as pollen grains must adhere to its surface in order to complete pollination. When stigmata provide greater receptive surface area, individual flowers can collect more pollen (i.e., the greater its stigmatic load), more ovules can be fertilized, and more seeds can be produced, leading to greater fitness. Stigma length is an

important component of receptive surface area. In both morphs the stigma is bifurcated and pollen adheres to both sides of each projection. No significant difference between pin and thrum flowers was found in stigma lengths, which suggested that, at least in this aspect of female reproductive function, the morphs might be equally fit. However, reflective of the developmental differences between the morphs (see chapter 5), stigma length correlated more strongly with the anther characteristics in pin flowers than in thrum flowers, presumably because pin stigmas continue to grow until they reach the mouth of the corolla tube while thrum stigmas stop growing earlier and closer to the base.

The stigma is not a flat surface, however. Stigmatic papillae both increase the surface area of the stigma and provide grooves in which pollen grains fit. Pin papillae were significantly longer than thrum papillae meaning that short-styled flowers have short papillae and long-styled flowers have long papillae. In the current model for the heterostyly supergene in *Primula* (Richards 1997), the characteristics of the female reproduction organ (gynoecium), including stigma height and length, are located on the same gene. And, this same mechanism likely controls papillae height. In *H. caerulea*, stigma length correlated strongly with stigma height.

Like papillae, pollen diameter showed intermorph variation; pin pollen was smaller than thrum pollen. In *H. caerulea*, the thrum:pin pollen diameter ratio found in northeast Ohio populations was 1.20, the same value reported by Ornduff (1977) and Wyatt and Hellwig (1979). This difference suggests a symmetry in regards to the size of pollen and papillae that interact during a legitimate pollen exchange. In a legitimate pollination, the smaller pin pollen interacts with the smaller thrum papillae, and the larger thrum pollen interacts with the larger pin papillae. Also, the average diameter of thrum

pollen is equal to the average length of thrum papillae. The functional significance of these relationships is unclear, however. Darwin (1877) suggested that thrum pollen is larger because the pathway down the longer pin style would require a greater store of nutrients, a premise invalidated by subsequent empirical evidence (Richards 1997). In the *Primula* model of the heterostyly supergene, pollen size and development of the male reproductive organ (androecium) are controlled by separate genes (Richards 1997). In *H. caerulea*, long-styled flowers produced smaller pollen grains than short-styled flowers, which suggests that style-length and pollen size may be controlled by different genes.

Among each of these traits predicted to relate to fitness – corolla tube shape, stigma morphology, and pollen size– variation across the populations sampled was almost always significant and generally considerable, as is common with fitness components (Price and Schluter 1991; Houle 1998). For example, Conner et al. (2003) quantified levels of phenotypic variation in many of these traits within radishes, noting high variance levels both in greenhouse experiments and in field approaches, although high variance levels from the field collections largely precluded separation of genetic and environmental influences to the traits. The variation reported here in *H. caerulea* was expressed either as differences among the means from the populations within pins and thrums, or as interaction effects, which means larger/smaller sizes in one morph were not necessarily the larger/smaller in the alternate morph. Population differences are commonly considered to reflect evolvability, or the possibility for the traits to change over time (Hansen et al., 2003). Although these traits were sampled from different localities all from a small region with various parks totaling 21,000 acres, these

populations may be isolated. Urban lands separate most without obvious corridors for genetic exchange.

Why does so much variation exist? Fisher (1930) suggested that fitness-relevant traits under selection should exhaust additive genetic variation. Variation among field populations certainly confound phenotypic plasticity and possible genetic variation, and variation present may combine dominant, pleiotropic and epistatic events to additive variation typically used to estimate heritability (Kelly 2005; Koczorowski et al. 2008). A trade-off must be present in the distylous mating system, as selection has favored two stable flower forms, the pins and the thrums, although the differences in anther and stigma heights a are not precisely reciprocal (Chapter 2). Variation in ancillary traits discussed here support that the dimorphisms have not yet reached fixation, and therefore flowers likely vary and may shift evolutionarily in response to subtle but largely unidentified changes in selection pressures.

Quantitative genetics has shown the possibility for single traits to change rapidly in response to selection. Flower size (Lehtila and Brann 2007) and ancillary traits, such as pollen size (Lamborn et al. 2005) will likewise respond rapidly to direct selection. No one has yet tried to select concurrently on a suite of floral characters, as in distyly, and therefore understanding how so much variation can be sustained in this system must await future experimentation in a more tractable species.

CHAPTER IV

FUNCTIONAL STATUS OF DISTYLY IN *HEDYOTIS CAERULEA*

Abstract

The mating system of a species is a dynamic property subject to modification in response to changes in selective pressures. Where a breakdown of distyly has been documented, changes have included the development of completely separate sexes (dioecy), or, more frequently, the development of a monomorphy consisting of self-fertile homostyles. Twelve populations of *Hedyotis caerulea* in or near the Cleveland Metroparks were surveyed in order to evaluate the functional status of distyly as a mating system of this species. Morph frequency was assessed and pollen production was used as a measure of male fitness by estimating the number of pollen grains per anther sac in a sample from four populations. The number of seeds per mature capsule was estimated from independent samples in three populations, which served as a measure of maternal fitness. All populations surveyed were isoplethic (i.e., contained a morph ratio of pins to thrums that was not different from 1:1), a result predicted when the species' mating system promotes intermorph pollen transfer (dissortative mating) with nearly 100% outcrossing. Pin flowers were found to produce significantly more pollen than thrum

flowers. However, pin flowers produced significantly smaller pollen than thrum flowers. Therefore, if volume of pollen grains produced is considered a measure of investment, pins and thrums appear to be allocating roughly the same amount of resources to pollen production and appear to have equal male fitness. No significant difference in seed set was found between pins and thrums, suggesting that female fitness also is equal**.** The ratios and similarity in fitness between morphs supports that the SI system in *H. caerulea* is stable.

Introduction

The mating system of a species is a dynamic property subject to modification in response to changes in selective pressures (Barrett and Eckert 1990), and distyly is no exception. The breakdown of distyly has been documented in several families, either through the development of completely separate sexes (dioecy), or, more frequently, through the development of a monomorphy consisting of self-fertile homostyles. The possibility of the first type of breakdown, gender specialization, was recognized by Darwin (1877). The replacement of distylous, self-incompatible hermaphrodites by male and female flowers has traditionally been explained by a persistent pattern of asymmetrical pollen flow between morphs sustained over evolutionary time (Ganders 1974; Nicholls 1986; Feinsinger and Busby 1987; Stone 1996; Ree 1997; Nishihiro and Washitani 1998). In the most common pattern of asymmetry, pin morphs receive more compatible pollen and have greater female fitness than thrum morphs (Lau and Bosque 2003). Thus, pin morphs presumably could have become the female sex while thrums became the male sex, a scenario that has been proposed to explain the evolution of dioecy from distyly in many species (Ornduff 1966, 1970; Barrett 1980; Opler et al. 1975; Lloyd

1979b; Baker 1958; Beach and Bawa 1980; Vuilleumier 1967; Bir Bahadur 1968; Opler et al 1975; Ornduff 1966; Ganders 1979; Wyatt and Hellwig 1983; Pailler et al. 1998; Naiki and Kato 1999). However, dioecy is also believed to have resulted from the opposite pattern of asymmetrical pollen flower in at least four species (Feinsinger and Busby 1987; Stone 1996; Hicks et al. 1985; Lau and Bosque 2003). In all cases, the transition from distyly to dioecy is inferred by comparing closely related taxa.

More frequently, however, distyly has broken down in the direction of increasingly associative mating through weaker selection for outbreeding, changes that have been reported in several genera (Ganders 1979; Barrett 1988, 1992; Schoen et al. 1997). In general, the shift from obligate outbreeding to predominant self-fertilization is one of the major pathways of mating system evolution in flowering plants (Stebbins 1957, 1974; Baker 1959; Jain 1976). In distylous species, distinct morphs have been replaced by self-compatible homostyles in which the proximity of male and female sex organs within individual flowers facilitates self-pollination (Baker 1959, 1966), and in which the self-incompatibility system has broken down (Sakai and Wright 2008), possibly due to decreases in pollinator availability (e.g. Washitani et al. 1994; Pauw 2005) or population size (e.g., Ganders 1975).

Plant mating systems are measured (Barrett and Eckert 1990) by evaluating the functional status of various parameters in a species' natural environment through a series of investigations, including (1) determination of morph ratios of naturally occurring populations, (2) assessment of the degree of self-compatibility of the species, and (3) a comparison of the functional gender of the two morphs. First, studies of morph frequency in distylous plants can provide information on the dynamics of selection on

genes controlling mating systems. Since distyly promotes outcrossing through intermorph pollen transfer (disassortative mating), it is expected to result in populations with a stable equilibrium state of 1:1 morph frequencies (isoplethy), ready availability of compatible pollen, and optimal reproductive success (Barrett 1992; Matsumura and Washitani 2000; Kery et al. 2003; Shibayama and Kadono 2003). Heuch (1979) has shown theoretically that, provided no fitness differences among the style morphs exist, an isoplethic equilibrium is the only possible condition in large populations with disassortative mating (i.e., in distylous plant species). In distylous populations where morph frequencies are unequal, several possible factors may be involved including clonal propagation, founder effects, mating asymmetries among the morphs, relaxation of SI, or breakdown of distyly. Second, the degree of self-compatibility in a species is critical when characterizing a plant mating system because SI is often incomplete and varying degrees of seed set are produced after self-pollination (Lloyd and Schoen 1992). The degree of self-compatibility permitted by the SI system is assessed through controlled pollination programs involving the comparison of the seed set following self, intramorph, and intermorph pollinations (Schoen and Lloyd 1992). The resulting data can be used to calculate a self-compatibility index (Becerra and Lloyd 1992), which is the average seed or fruit set after self-pollination divided by the seed or fruit set after cross-pollination. The SC index can range from nearly zero to more than one, reflecting the continuous nature of self-compatibility. Third, hermaphroditic plants can obtain fitness by pollen donation (male fitness) as well as by fruit and seed production (female fitness). A plant that allocates its resources disproportionately to pollen production and dispersal would be considered functionally male, and a plant that allocates its resources to ovule production,

fertilization, and seed maturation would be considered functionally female. Since the resources available for sexual reproduction are finite, an increase in resource allocation to male function must be accompanied by a decrease in resource allocation to female function, and vice versa. Defining male fitness as α and female fitness as β, then theoretically the equilibrium of α, β is the pair which maximizes the product α β (Sutherland and Delph 1984). A fully distylous species would be made of individuals with equal male and female fitness, and would be typified by equivalent amounts of pollen from each morph pollinating the stigmas of the opposite morph. The functional gender of plants can be estimated by following pollen flow in natural populations and by counting the number of fruits and seeds a plant produces (Sutherland and Delph 1984).

The functional components of *Hedyotis caerulea* have been understudied. Morph frequencies in populations of this species have been quantified and published by three researchers: Ornduff (1977, 1980), who studied 19 populations in North Carolina over a period of several successive years, Wyatt and Hellwig (1979), who studied six populations in North Carolina; and Grimaldi (1988), who reported that pins slightly outnumbered the thrums in several New York populations. The results of controlled pollination programs have also been published by Ornduff (1977, 1980) and Wyatt and Hellwig (1979). Ornduff (1977) measured pollen flow and seed set in natural populations of *H. caerulea*, and he found that although compatible pollen flow from pins to thrums was greater than from thrums to pins, seed production by the two morphs was equal. Based on observations in the field, Ornduff (1980) and Wyatt and Hellwig (1979) suggested that, while the pin and thrum morphs may produce the same number of buds,

flowers, and capsules per plant, it is possible that differences in these morphs may occur across populations or at certain times of the flowering period.

In order to evaluate the functional status of distyly in naturally occurring populations of *H. caerulea* in northeastern Ohio, this study focused on morph ratios, the calculated species' self-compatibility index, and the functional gender of the two morphs. In order to ascertain any temporal component of the mating system, several populations were surveyed at multiple times throughout the flowering period.

Materials and Methods

Twelve populations of *H. caerulea* in or near the Cleveland Metroparks were surveyed in May of 2005 (see Table 4.1). Ten of these populations also were sampled for measurement of floral characters (see chapters 1 and 2). For each population, one flower from each individual plant was collected. The flowers were then taken back to the lab, separated and counted by morph. Morph frequencies were compared using a chi-square test.

Four populations were surveyed every two weeks over the 2008 growing season (i.e., on May 7, May 18, June 1, and June 14). Populations surveyed included # 1 (in Brecksville), # 13 (a previously unstudied population in Bedford), # 7A and # 7B (population # 7 subdivided into two smaller populations; see Table 2.1). In each survey the following were counted: the number of individual pins and thrums, the number of flowers per individual pin and thrum, and the number of capsules per individual pin and thrum. Due to the difficulty in finding and identifying the morph of a plant without at least one flower, only flowering individuals were counted.

Pollen count per anther sac was estimated in a third sampling of four of these populations (locality # 14 in the Brecksville, locality # 10 in North Chagrin, locality # 2 in Bedford, and locality # 9 in Rocky River) in 2008. Ten pin flowers and ten thrum flowers were collected from each site. One of the four anthers from each flower was placed in a small plastic vial with 70% ethanol, 2 drops of methylene blue, and one drop of detergent, for a total volume of one milliliter. The pollen was suspended throughout the liquid in the vial using a vortex mixer. The pollen grains contained within 0.1 mL of the suspension were scored using a microscope (Leitz Dialux EB20) and a slide equipped with a grid. Total pollen count was calculated for each anther sac.

In June 2008, forty mature capsules from pin plants and forty mature capsules from thrum plants were collected from each of three populations (#2 in Bedford, # 10 in North Chagrin, and # 14, a population in the Brecksville Reservation that had not been sampled previously). In the lab each capsule was opened using a dissecting needle and the seeds were counted under a dissecting microscope.

Attempts to determine the degree of self-incompatilibity in *Hedyotis caerulea* through controlled pollinations were made in site # 2 (see Table 2.1) during three consecutive growing seasons. In May and June 2005 a total of 60 previously unpollinated flowers were hand pollinated in the field using a dissecting needle (Ornduff 1980) and a field magnifier and covered with parafilm (Mal and Lovett-Doust 1997) to prevent further pollination. Since mature buds could be forced open with a tap of a probe, selecting them for the study precluded previous pollination. Five pins and five thrums served as controls (covered without pollination); ten pins and ten thrums were pollinated legitimately; five pins and five thrums were self-pollinated; five pins were

pollinated with pin pollen from other pin flowers; five thrums were pollinated with thrum pollen from other thrum flowers. In May and June 2006 the study was repeated. In May and June 2007 the study was again repeated with modification; the number of flowers pollinated was doubled, and half of the pollinated flowers were covered with parafilm, as done previously, and half were enclosed in a tent of gauze to prevent visitation by pollinators but to allow circulation of air. A series of pollinations were also done in the lab by collecting mature buds and carrying out the pollination program under a dissecting microscope, staining according to Kearns and Inouye (1993) and observing the gynoecium for growth of a pollen tube via fluorescence microscopy (Leitz Dialux EB20).

Results

The twelve populations surveyed were generally found in two types of settings: along forest trails (populations 1, 3, 4, 5, 6, 8, 12; see Table 4.1) and on the edges of picnic areas (populations 7, 9, 10) in the Cleveland Metroparks. The trails tended to follow the edges of ridges, which allowed nearly full sun part of the day and diffuse sun part of the day. Populations in picnic areas were exposed to full sun a greater part of the day, and appeared to experience less competition for light from other plants in the community. Population density appeared to be greater in picnic areas than along trails. Populations 2 and 11 did not fit into either category: population 2 was located in a large field in Bedford Reservation, while population 11 was located on the back lawn of privately owned property near the Metroparks, and both received full sun all day.

Population size ranged from 68 to 913 individuals, with a mean size of 404 (see Table 4.1). Chi square tests indicated that, with the exception of population 3, the morph ratio of pins to thrums was not different from 1:1, which made these populations

isoplethic. Population 3, which was the smallest population, was anisoplethic with a pin to thrum ratio of 2.6:1 ($P < 0.05$), but not significant at an experiment wide level (Rice 1989). In an independent follow-up survey in 2008, population 3 was isoplethic.

The results of season-long surveys done in two trail and two picnic area populations are given in Figures 4.1, 4.2, 4.3, and 4.4. In all four populations there were no statistical differences in the number of pin and thrum plants across the flowering season with the exception of the third survey of population 1 in which pin plants outnumbered thrum plants ($P < 0.01$; see figure 4.3). The number of pin flowers was also statistically not different from the number of thrum flowers, with the following exceptions in which pin flowers outnumbered thrum flowers: the second survey of population 6b ($P < 0.001$; see figure 4.2); the third survey of population 1 ($P < 0.05$; see Figure 4.3); and the first survey of population 13 ($P < 0.001$; see figure 4.4). With one exception, there was no statistical difference between the number of pin capsules collected and the number of thrum capsules collected; more pin than thrum capsules were found in the third survey of population 13 ($P < 0.05$; see figure 4.4).

Across four populations, the mean pollen count was 856 ± 67.4 pollen grains per anther sac in pin flowers and 583 ± 49.8 per anther sac in thrum flowers. Pins therefore produced significantly more pollen grains than thrums ($P < 0.001$). Mean pollen counts per population also varied, and ranged from 752 ± 181 to 937 ± 93.7 in pins and from 483 ± 55.2 to 672 ± 130 in thrums (see Figure 4.5), giving a significant locality effect ($P = 0.012$).

Analysis of seed number produced per capsule showed a small but significant locality effect ($P < 0.05$), but no intermorph variation ($P = 0.67$, n.s.). On average, pin

Table 4.1 Results of a survey of twelve populations of *Hedyotis caerulea* in the greater Cleveland area, mostly in the Cleveland Metroparks. The morph ratio of pins to thrums was calculated for each population.

Site #	Location	N	# pins	# thrums	X^2	Morph Ratio
1	Buckeye Trail Brecksville Reservation	326	169	157	0.22	1:1
2	Emerald Circle Prairie Bedford Reservation	338	156	182	1	1:1
3	Girl's Scout Camp Bedford Reservation	68	49	19	4.97	2.6:1
4	Willis Picnic Area Bedford Reservation	543	275	268	0.045	1:1
5	Hemlock Creek Bedford Reservation	883	443	440	0.046	1:1
6	Hermit Hollow Bedford Reservation	254	122	132	0.20	1:1
7	Egbert (population 1) Bedford Reservation	89	42	47	0.14	1:1
8	Egbert (population 2) Bedford Reservation	200	105	95	0.25	1:1
9	Big Cedar Area Rocky River Reservation	825	407	418	0.073	1:1
10	Strawberry Hill Picnic Area North Chagrin Reservation	913	466	447	0.22	1:1
11	Howe Road, Strongsville (private property)	266	127	139	0.27	1:1
12	State Road, Hinckley (private property)	147	81	66	0.76	1:1

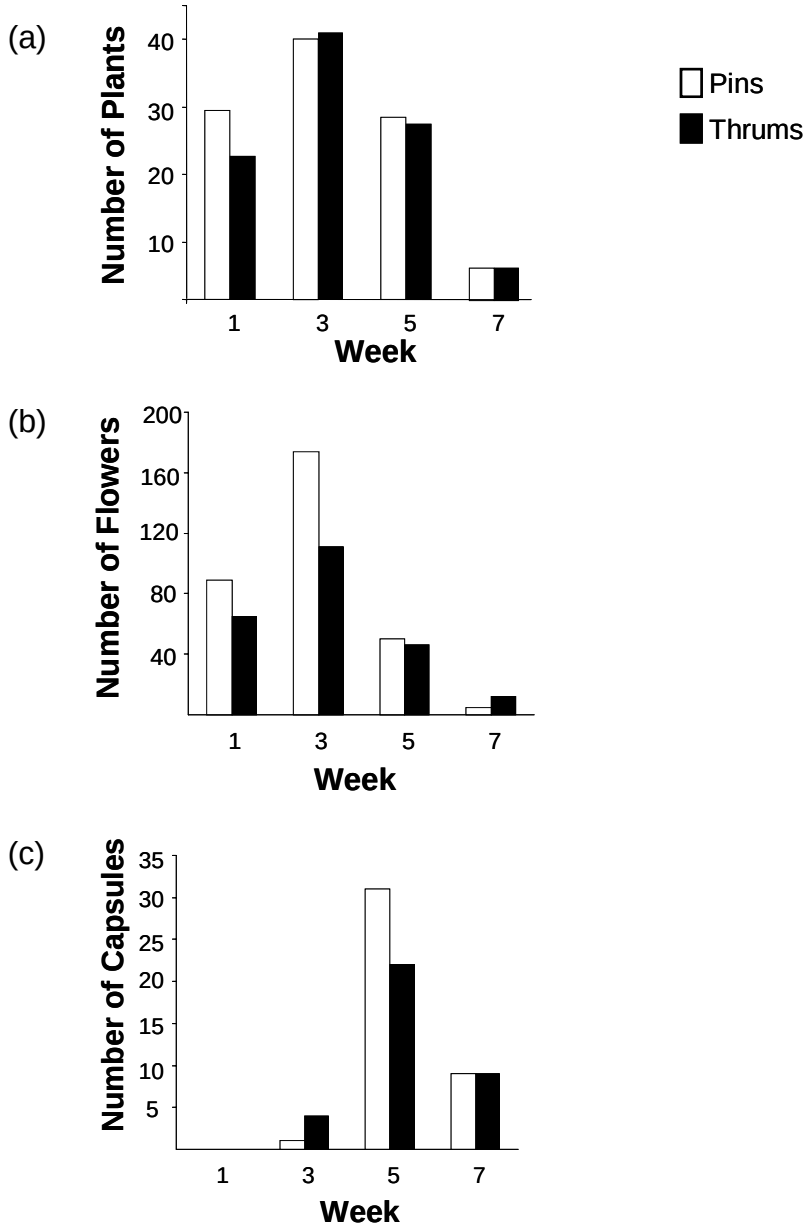

Figure 4.1 The results of four successive surveys of the bluet population in locality 7a in the Bedford Reservation over a single growing season. For each survey, the number of individual plants, the number of flowers and the number of capsules were counted for each morph. White columns represent pins, and black columns represent thrums.

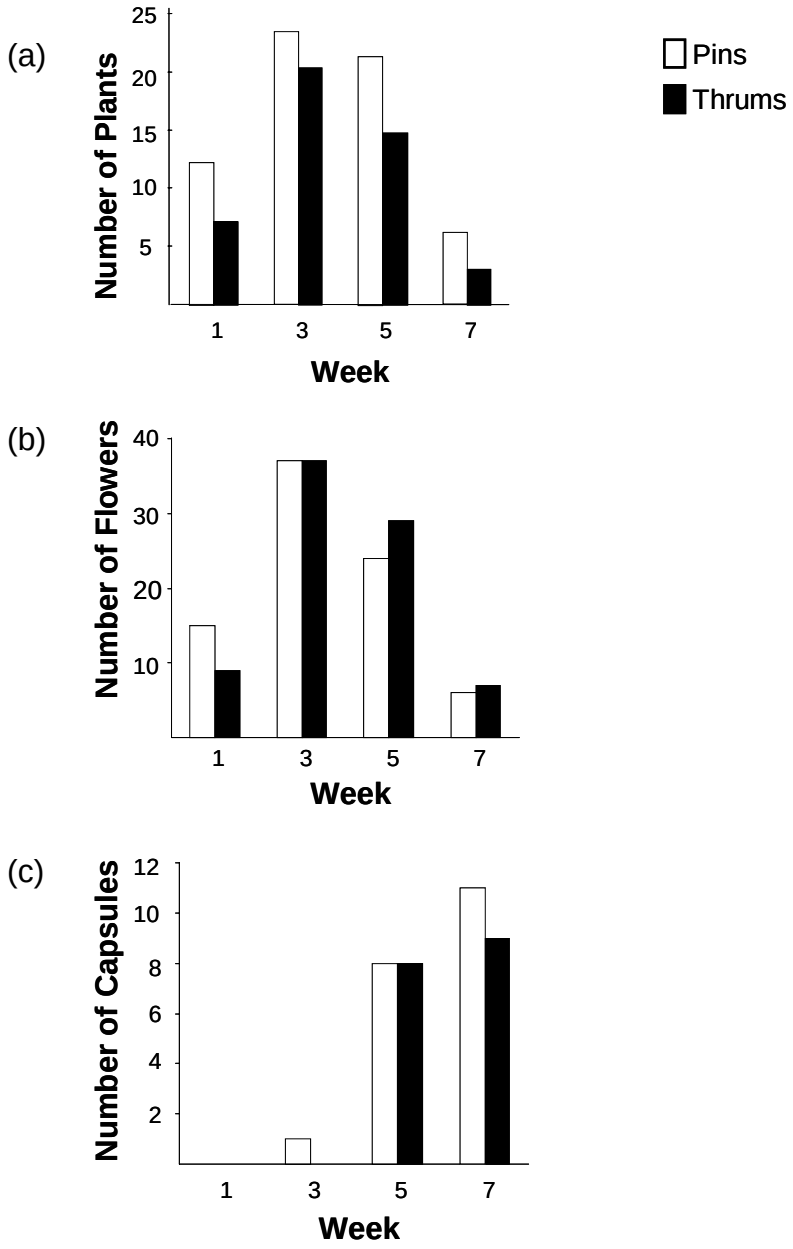

Figure 4.2 The results of four successive surveys of the bluet population in locality 7b in the Bedford Reservation over a single growing season. For each survey, the number of individual plants (a), the number of flowers (b), and the number of capsules (c) were counted for each morph. White columns represent pins, and black columns represent thrums.

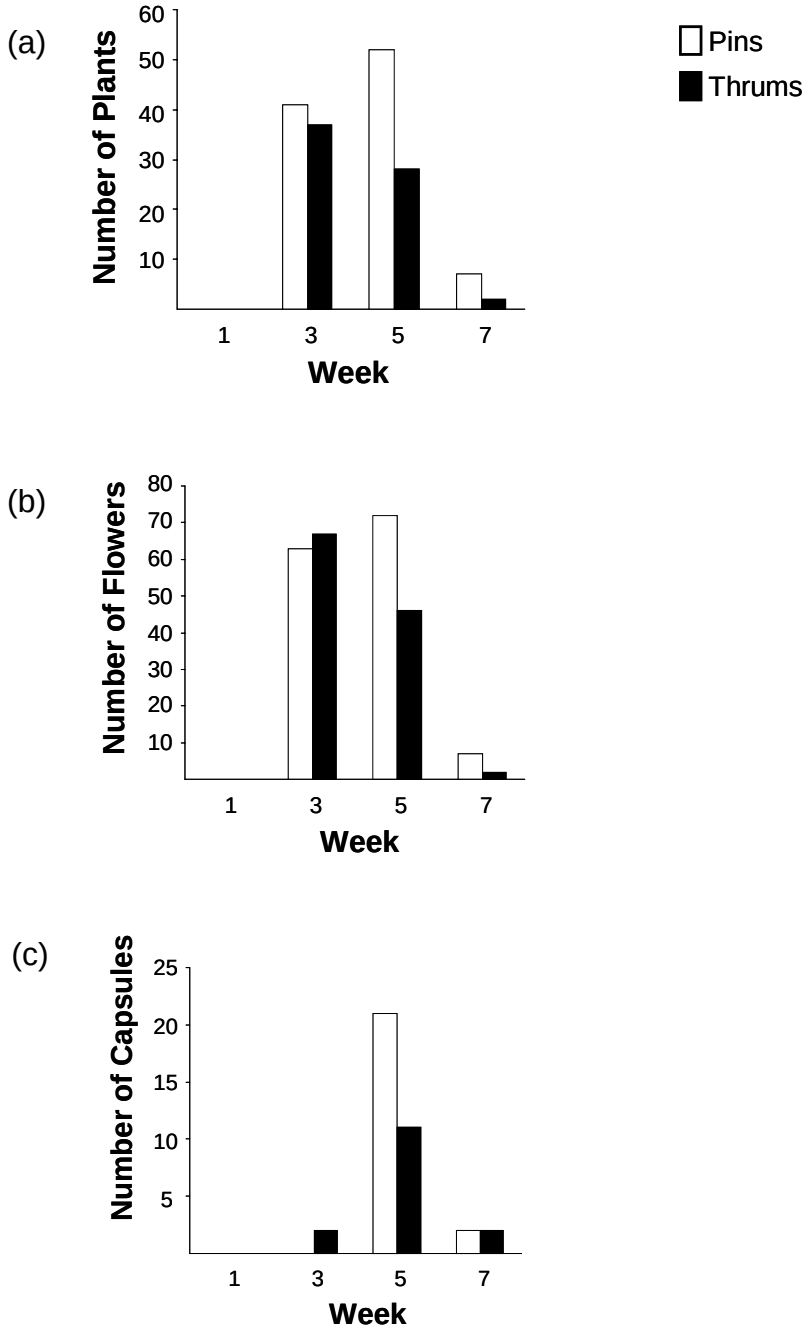

Figure 4.3 The results of four successive surveys of the bluet population in locality 10 in the Brecksville Reservation over a single growing season. For each survey, the number of individual plants (a), the number of flowers (b), and the number of capsules (c) were counted for each morph. White columns represent pins, and black columns represent thrums.

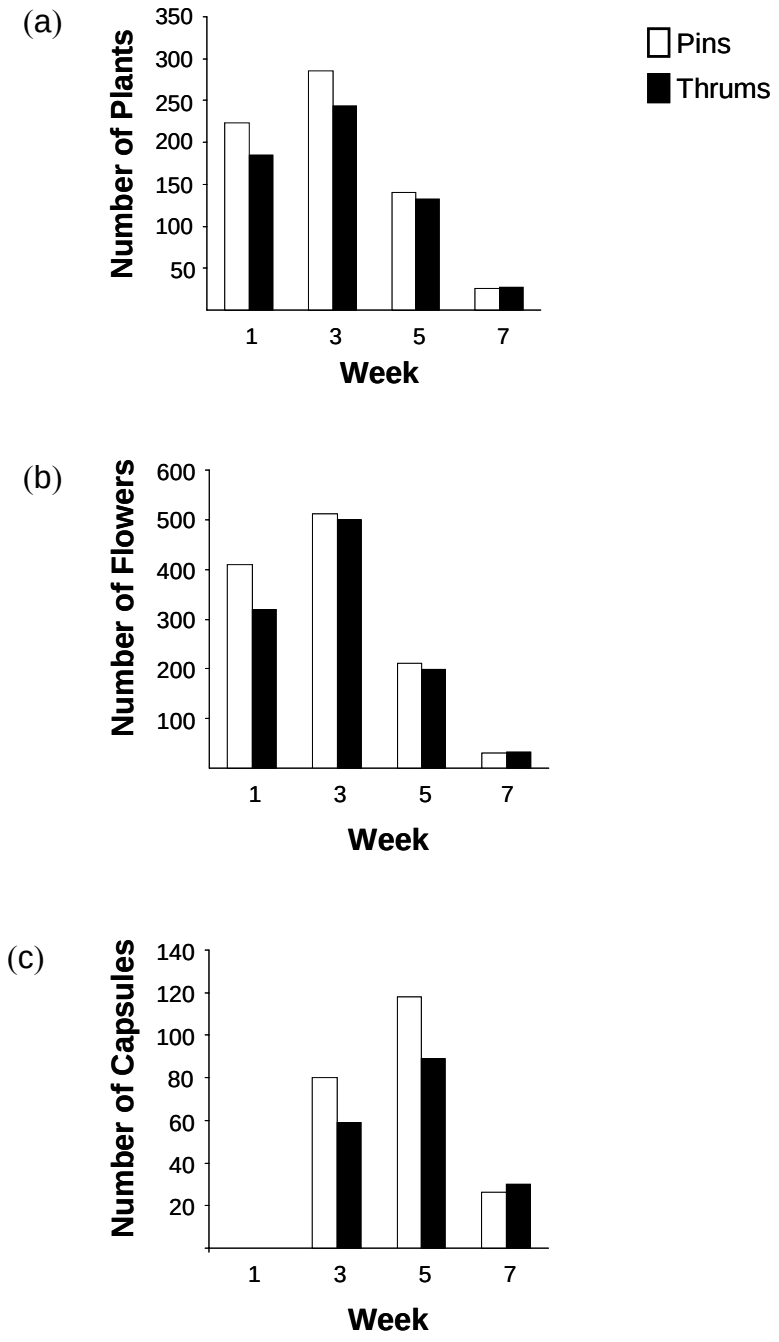

Figure 4.4 The results of four successive surveys of the bluet population in locality 13 in Bedford over a single growing season. For each survey, the number of individual plants (a), the number of flowers (b), and the number of capsules (c) were counted for each morph. White columns represent pins, and black columns represent thrums.

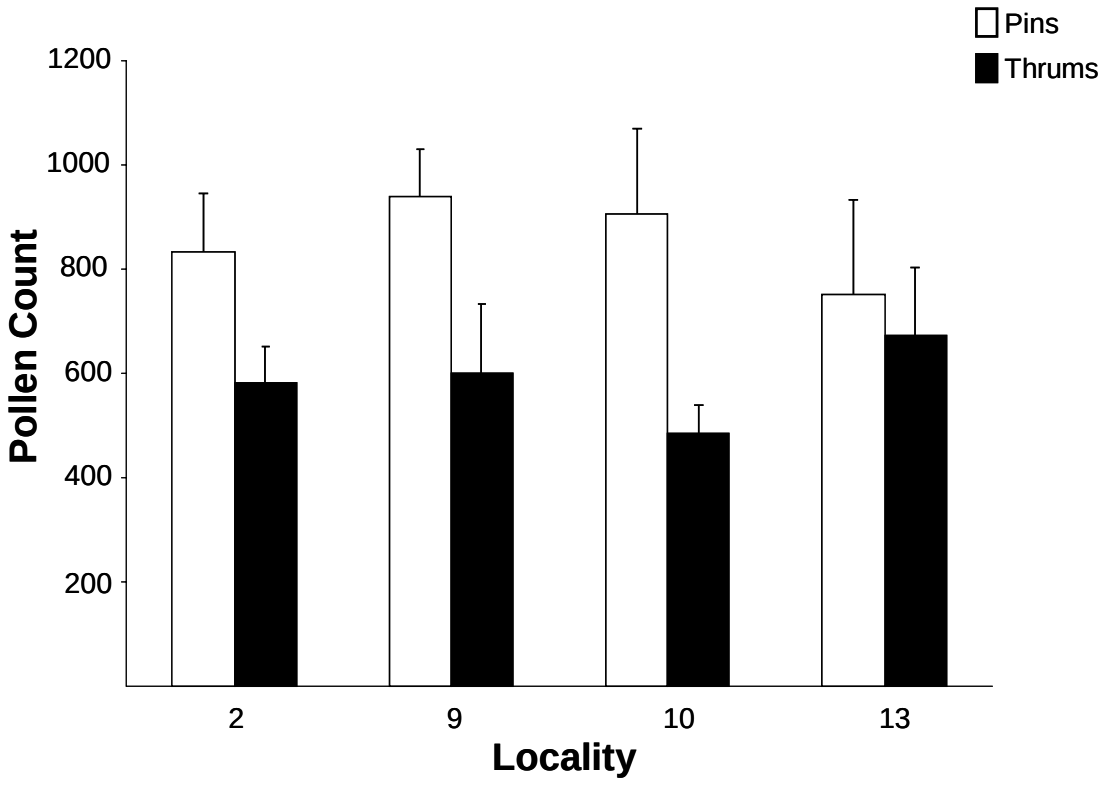

Figure 4.5 Pollen counts per single anther sac of *Hedyotis caerulea* pin and thrum flowers collected from four populations in the Cleveland Metroparks. Error bars represent standard errors.

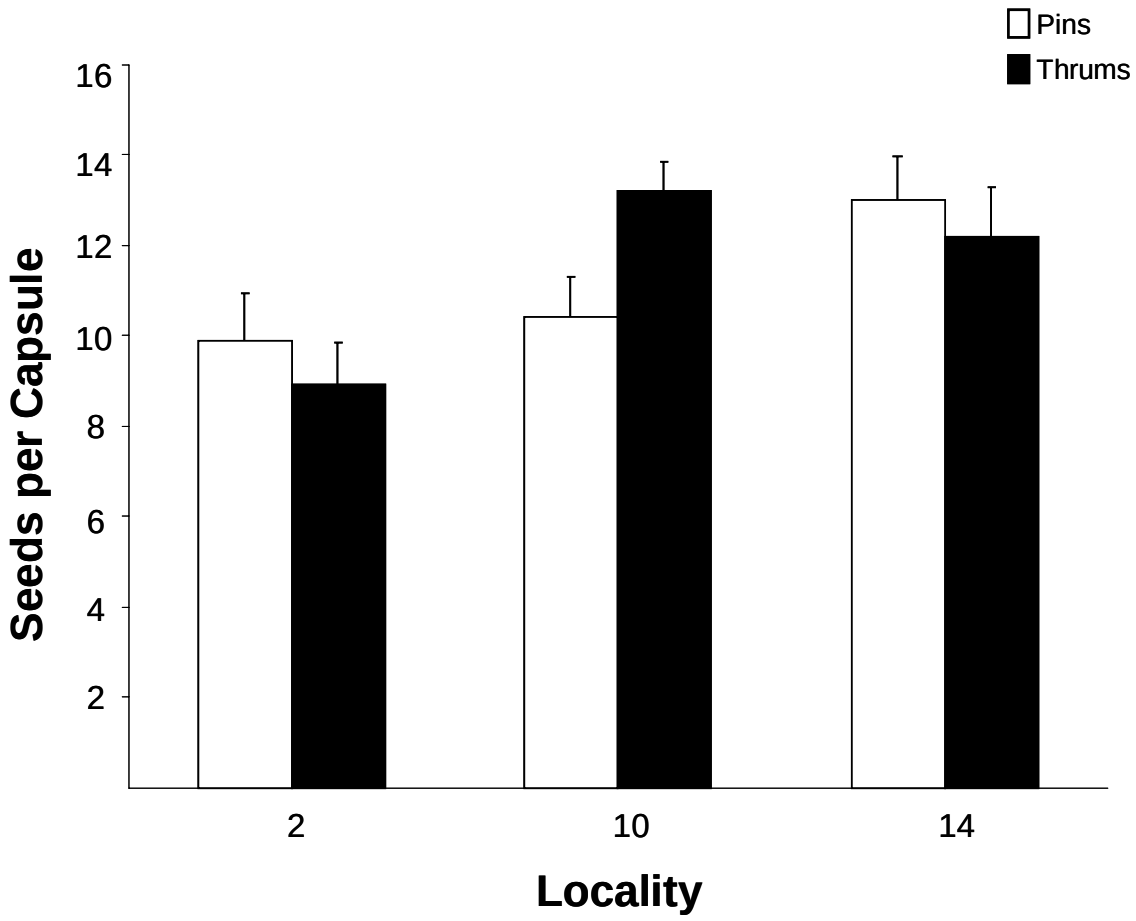

Figure 4.6 Seed counts per capsule collected from *Hedyotis caerulea* pin and thrum flowers collected from three populations in the Cleveland Metroparks. Error bars represent standard errors.

capsules produced 11.0 ± 0.58 seeds and thrum capsules produced 11.5 ± 0.54 seeds (see Figure 4.6).

Despite repeated attempts, controlled pollination experiments failed, first in 2006 due to vandalism of the field site, but in other attempts due to the delicate nature of the species. The flowers were very small, difficult to manipulate in the field, and easily damaged by attempts at controlled pollen transfer, attempts to isolate the flower to prevent uncontrolled pollination, and simply by attempts to label the plants so each could be located and identified at the end of the study. Controlled bud pollinations under a microscope in the laboratory failed to produce identifiable pollen tubes.

Discussion

All populations surveyed were isoplethic, with the possible exception of population 3 (the smallest one, N = 68). Considering that the experiment included ten populations, it was not unexpected to find one anisoplethic population by chance. Small populations of distylous species often show skewed morph ratios as a result of genetic drift (e.g. Eckert and Barrett 1992; Husband and Barrett 1992; Endels et al. 2002; Kery et al 2003), and population 3 had reached isoplethy when surveyed again three years later. An equal, 1:1 balance of morph frequencies in a population is achieved when the species' mating system promotes intermorph pollen transfer (dissortative mating) with nearly 100% outcrossing (Barrett, 1992; Van Rossum et al. 2006), and when the two morphs are of equal fitness. Thus, it is assumed that an isoplethic population offers both morphs equally prolific reproductive success through optimal availability of compatible pollen (Matsumura and Washitani 2000; Kery et al. 2003; Shibayama and Kadono 2003). Surveys of *H. caerulea* populations previously done in North Carolina (Wyatt and

Hellwig 1979; Ornduff 1977, 1980) found greater variation in morph frequencies per population as pins outnumbered thrums by a ratio of 1.5:1 in most of the populations he surveyed. While Wyatt and Hellwig (1979) also found that pins frequently outnumbered thrums, when they pooled the data from all populations surveyed the morph frequency was deemed isoplethic. The suggestion made by Wyatt and Hellwig (1979) and Ornduff (1977; 1980) that morph frequencies may fluctuate in some predictable pattern over the course of a flowering season was not supported empirically in the present study. While some variation was detected, three of the four populations studied for temporal effects showed no morph-specific differences in any time period, and while several showed differences in total flowers, there was no consistent pattern with respect to the growing season. Thus, any temporal difference in morph frequency is probably insignificant to the mating system.

As stated in Chapter 3, pin flowers produce significantly smaller pollen than thrum flowers, but they also produce significantly more pollen than thrum flowers. This inverse correlation between pollen size and pollen production has been shown in a number of distylous species (Ganders 1979; Dulberger 1992; Richards 1996). If male fitness were measured strictly by the number of pollen grains produced, pins would have the greater estimate of fitness. However, when the total volume of individual pollen grains produced was estimated for populations 1, 2, 9, and 10, based on average pollen counts and average pollen diameters from samples taken from those populations, the intermorph difference was not statistically significant ($P=0.11$). Therefore, if volume of pollen grains produced is considered a second measure of investment, pins and thrums

appear to be allocating roughly the same amount of resources to pollen production and appear to have equal male fitness.

Another measure of male fitness is pollen flow. Ornduff (1980) reported a somewhat skewed pattern of pollination in natural populations in North Carolina, specifically lower levels of intermorph pollen flow and higher levels of intramorph pollen flow than expected. He came to this conclusion by collecting naturally pollinated flowers from the field, examining the stigmas microscopically, and scoring the number of pin and thrum pollen grains on each stigma, using pollen size to distinguish the morph that had created each pollen grain. He reported that the number of pin pollen grains mostly outnumbered the thrum pollen grains when he examined pin stigmas. This procedure was not used with the Ohio bluets because of the overlap in diameters of pin and thrum pollen, larger than Ornduff found. However, given the balanced morph ratios of the populations surveyed here, any differences in pollen flow have not affected morph frequency.

Female fitness is frequently measured as seed set. No significant differences in seed set were found between pins and morphs, suggesting equal female fitness. These findings support sex allocation theory (Sutherland and Delph 1984), which predicts that, since resources for sexual reproduction are finite, any increase in resource allocation to male function must be accompanied by a decrease in resource allocation to female function and vice versa. Defining male fitness as α and female fitness β, then it can be theoretically demonstrated that, under natural selection, the equilibrium of α, β is the pair which maximizes the product $\alpha\ \beta$. In *H. caerulea*, all evidence suggests that $\alpha_{pins} =$

α_{thrums}, and $\beta_{pins} = \beta_{thrums}$. It follows, then, that this species shows no movement towards dioecy in northeastern Ohio.

This study offers no new results from controlled pollinations. Previous studies indicate, however, that *H. caerulea* has a strong self-incompatibility system that strictly enforces outcrossing. In a program involving plants transplanted from the field into pots in a greenhouse, Ornduff (1977, 1980) conducted a total of 560 self-, intramorph, and intermorph pollinations. He reported that self-pollinated pins produced an average of 0.1 seeds per pollination and that self-pollinated thrums produced 1.0 seeds per pollination. Pollinations between different plants of the same floral morph are also relatively unproductive; pin x pin pollinations produced 0 seeds; thrum x thrum pollinations produced an average of 1.1 seeds. Pollinations between pins and thrums produced an average of 23.0 seeds per pollination. In northeastern Ohio under natural field conditions pins and thrums produced approximately half that number of seeds per pollination.

Thus, mating within each group is essentially non-existent; sexual reproduction likely occurs only as a result of mating between groups. Wyatt and Hellwig (1979) conducted a smaller study that involved 48 incompatible pollinations that resulted in 2% success in producing seed and 20 compatible pollinations that resulted in 95% success in producing seed. To date no report has been published that disputes the existence of a fully functioning SI system in *H. caerulea*, a conclusion that is supported by the isoplethic morph ratios found in Ohio. Just as there is no evidence to indicate a movement towards dioecy, there no indication that the SI system is undergoing a break

down that would be necessary for the species to move towards selfing or homostyly. Distyly in *H. caerulea* is apparently a stable, functional system.

CHAPTER V

DEVELOPMENTAL ASPECTS OF DISTYLY IN *HEDYOTIS CAERULEA*

Abstract

Studies of floral development have made significant contributions to the understanding of the evolution of plant mating systems. However, evolutionary events leading to distyly remain obscure because few detailed studies on early floral development exist. Here the initiation, rate, and termination of growth between the stamens and stigmas in *Hedyotis caerulea* were quantitatively compared to determine when and how floral morphology of pins and thrums diverge. Buds at multiple stages of development were collected from five populations of *Hedyotis caerulea* in the Cleveland Metroparks. Each bud was then measured for length, opened, and the heights of these developing stigmas and anthers were compared against total bud length. Pins and thrums were distinguishable when the bud length was less than one millimeter. The growth rate of anthers was uniform for both morphs, but thrum anthers grew faster than pin anthers, suggesting dimorphy in anther height developed from a significant difference in growth rates between anthers and whole buds. Dimorphy in stigma heights developed through a less straightforward mechanism. Pin stigmas grew at a faster rate early in development,

but in addition, late in bud development, the rate of thrum stylar growth slowed, which resulted in a curvilinear growth pattern for thrums. This growth pattern differed from that of another distylous *Hedyotis* species, *H. salzmannii*.

Introduction

Morphological variation in flowers is determined by the combined effects of genotype, developmental programming, and environmental factors (Diggle 1992). Not surprisingly then studies of floral development have made significant contributions to understanding the evolution of plant mating systems. When available, phylogenetic hypotheses concerning the taxa studied can be merged with variation in their developmental patterns to identify possible evolutionary pathways by which new morphologies emerged (e.g., Guerrant 1982; Hufford 1995; Kellogg 1990; Friedman and Carmichael 1998).

The understanding of the evolution of distyly has been hampered by a lack of data, particularly on floral development. Fewer than 20 papers during the last 100 years have critically examined how the different forms of flowers present in heterostylous species arose (Cohen 2008), and the majority of them focused on tristylous species (Faivre 2000; Hernandez and Ornelas 2007). In one review, Richards and Barrett (1992) concluded that variation in floral organ development among families derives from independent evolutionary events that led to tristyly. While they predicted that the same could be true for distyly, they lacked information on distylous floral development in a sufficient number of species to test this hypothesis (see Table 5.1).

Despite the limited number of distylous species studied, at least four distinct developmental pathways have been characterized for achieving the distinct difference

Table 5.1 Published studies on floral development in various distylous species.

Species	Family	Publication
Primula spp.	Primulaceae	Stirling 1932
Faramea suerrensis	Rubiaceae	Richards and Barrett 1992
Guettarda scabra	Rubiaceae	Richards and Koptur 1993
Quinchamalium chilense	Santalaceae	Riveros et al. 1995
Hedyotis salzmannii	Rubiaceae	Riveros et al. 1995
Psyochotria spp.	Rubiaceae	Faivre 2000
Bouvardia ternifolia	Rubiaceae	Faivre 2000
Primula vulgaris	Primulaceae	Webster and Gilmartin 2006
Linum spp.	Linaceae	Arbruster et al. 2006
Palicourea padifolia	Rubiaceae	Hernandez and Ornelas 2007
Lithospermum spp.	Boraginaceae	Cohen et al. 2008

observed in stigma height between pin and thrum flowers, and at least two developmental patterns contribute to differences in anther height between the morphs (Faivre 2000; Cohen 2010). It is therefore likely that additional developmental pathways will be identified as more distylous species are studied (Cohen 2010).

Here I quantitatively compared the initiation, rate, and termination of growth between the stamens and stigmas in the distylous *H. caerulea* in order to establish when and how the floral morphogy of pins and thrums diverged. These observations are compared to developmental patterns reported for other distylous species and to putative phylogenies of these taxa to produce inferences about the frequency and pattern by which distyly evolved.

Materials and Methods

In May 2008, 212 pin buds and 219 thrum buds at various stages of development were collected from five populations of *H. caerulea* in northeastern Ohio (populations #2 and #4 in Bedford; #9 in Rocky River; #10 in North Chagrin; and #13 in Brecksville). Buds were chosen in manner that included the widest range of development possible, although the smallest buds were selected first in order to ensure their adequate representation in the data set given a short growing season. All buds were preserved in 80% ethanol. Buds were dissected to measure bud length, stigma height, and anther height using an Olympus SZX12 dissecting microscope equipped with an ocular micrometer.

Bud size and floral organ measurements were log-transformed for analyses to normalize data. Size of developing stigmas and anthers were analyzed against total bud

length using linear regression analyses (Proc GLM; SAS Institute 2002) both with and without a second-order term in the model to determine if the relationship could best be described as curvilinear or linear. Bud length provided a relative estimate of time to which stigma height and anther height were compared in pin and thrum morphs.

Results

Differences in anther height made the pins and thrums distinguishable when the bud length was less than one millimeter, which indicated that variation in growth rates occurs very early in development (Fig. 5.1). In both morphs, the filament serves only to attach the anther to the inner surface of the corolla tube and made no contribution to the height of the anther. When the results from all five populations sampled were pooled and log-transformed, the linear term ($P < 0.0001$) explained most of the variation in the relationship between bud length and anther length in both pin ($r^2 = 0.877$) and thrum ($r^2 = 0.950$) flowers (see Tables 5.1, 5.2, 5.3, and 5.4). A second-order term did not increase r^2 significantly in either morph ($P = 0.175$, n.s.). Thus, the relative growth of bud length versus anther height in both pins and thrums was described by a best fit linear equation. The slope of these lines differed significantly between morphs, however, with a slope for the pin flowers of 0.43 ± 0.01, while that of the thrums was 0.69 ± 0.01 (see Tables 5.2 and 5.3). Importantly, these slopes also were significantly less than one [pins ($P<0.0001$); thrums ($P<0.0001$)].

Intermorph differences in stigma development were not as straightforward as those in anthers (see Fig. 5.2). The slope of bud length versus stigma height again differed significantly between morphs, but for stigma growth, shape as well as magnitude of growth rate varied. The slope based only on linear regression for the pin flowers was

0.97 ± 0.17, while that of the thrums was faster at 1.40 ± 0.11 (see Table 5.2). The relationship between bud length and stigma height for pins could be described solely by the linear term, ($r^2 = 0.836$, $P < 0.0001$), and a second-order term did not increase r^2 significantly ($P = 0.706$, n.s.). Thus, the relative growth of bud length versus stigma height in pin morphs was described as isometry, meaning the slope was not different from one.

This relationship in thrums possessed some curvilinear characteristics (see Tables 5.4 and 5.5). Both the linear ($P < 0.0001$) and the second-order ($P < 0.0001$) terms were necessary to describe the relationship between the development of buds and stigmas in thrum flowers. Growth began at a rate significantly faster than one (a slope of 2.44 ± 0.30 for the range 0-1.5 mm) and then tapered off significantly ($P < 0.0001$) for buds larger than 1.5 mm (to 0.38 ± 0.08).

Discussion

Anthers grew more slowly than buds as apparent from slopes significantly less than one in pins and thrums indicative of a negative allometric relationship with bud elongation in both morphs. The growth rate was uniform for both morphs, but thrum anthers grew faster than pin anthers. Thus, dimorphy in anther height developed from a significant difference in growth rates between anthers and whole buds beginning in early stages of bud elongation.

This relationship for the development of differences in anther size occurs in many of the distylous species studied thus far (Riveros et al 1987; Richards and Barrett 1992; Richards and Koptur 1993; Faivre 2000; Hernandez and Ornelas 2007; Cohen et al. 2008). In fact, the only other variation that has been observed for anthers is that, in

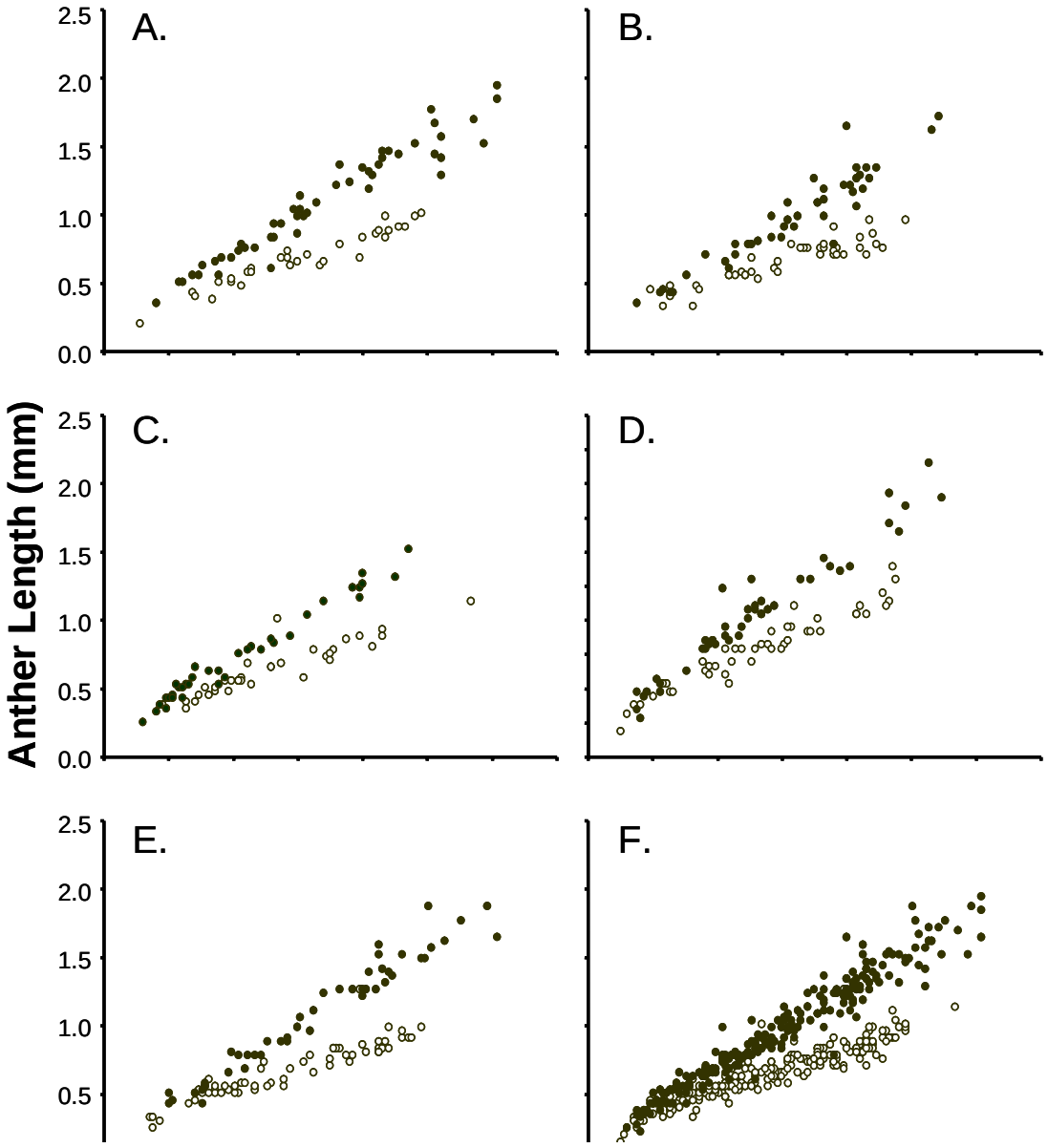

Figure 5.1 Anther development in pin and thrum floral morphs across five populations of *H. caerulea*: A = population 2; B = population 4; C = population 9; D = population 10; E = population 13; F = all populations combined. Data of bud lengths (mm) versus anther heights (mm) were plotted for each bud collected.

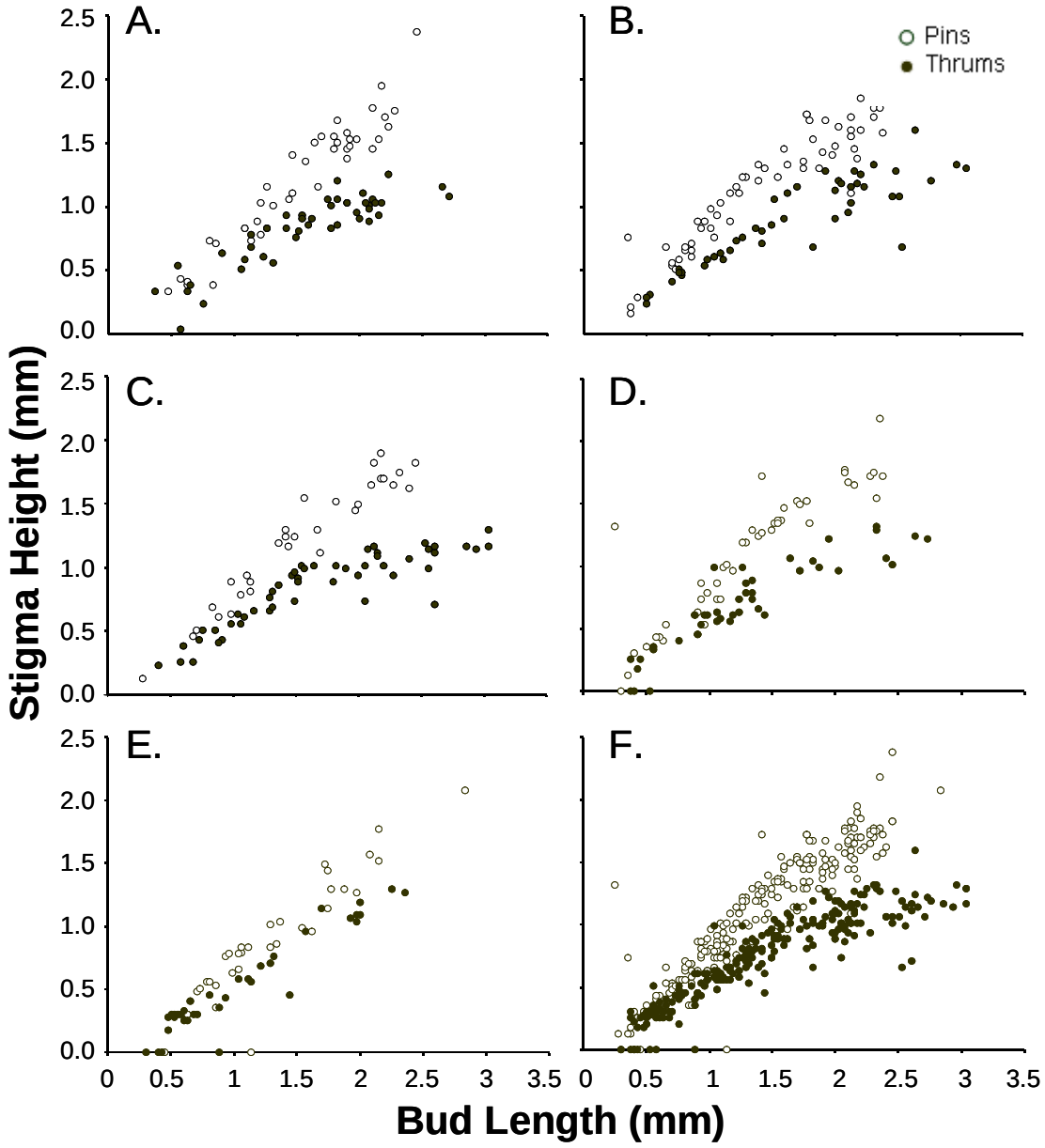

Figure 5.2 Stigma development in pin and thrum floral morphs across five populations of *H. caerulea*: A = population 2; B = population 4; C = population 9; D = population 10; E = population 13; F = all populations combined. Data of bud lengths (mm) versus stigma heights (mm) were plotted for each bud collected.

Table 5.2. Linear regression analysis of relative growth rate of anther heights against bud length and stigma heights against bud length for pin and thrum floral morphs across five populations of *H. caerulea*.

ANTHER HEIGHT

Pin

Locality	Slope	y-intercept	P	r^2	N
2	0.39 ± 0.03	0.15 ± 0.03	< 0.0001	0.835	42
4	0.42 ± 0.02	0.15 ± 0.02	< 0.0001	0.875	59
9	0.47 ± 0.02	0.08 ± 0.02	< 0.0001	0.937	31
10	0.47 ± 0.02	0.10 ± 0.02	< 0.0001	0.920	46
13	0.46 ± 0.03	0.12 ± 0.03	< 0.0001	0.855	34
Overall	0.43 ± 0.01	0.13 ± 0.01	< 0.0001	0.877	212

Thrum

2	0.69 ± 0.04	0.03 ± 0.03	< 0.0001	0.902	43
4	0.73 ± 0.02	0.03 ± 0.02	< 0.0001	0.962	47
9	0.69 ± 0.02	0.06 ± 0.02	< 0.0001	0.945	50
10	0.68 ± 0.02	0.06 ± 0.02	< 0.0001	0.953	43
13	0.67 ± 0.02	0.08 ± 0.01	< 0.0001	0.973	36
Overall	0.69 ± 0.01	0.05 ± 0.01	< 0.0001	0.950	219

STIGMA HEIGHT

Pin

Locality	Slope	y-intercept	P	r^2	N
2	1.00 ± 0.05	0.03 ± 0.04	< 0.0001	0.906	42
4	0.82 ± 0.04	-0.24 ± 0.03	< 0.0001	0.876	59
9	0.92 ± 0.04	-0.06 ± 0.04	< 0.0001	0.946	31
10	0.88 ± 0.07	-0.003±0.06	< 0.0001	0.788	46
13	1.02 ± 0.09	-0.22 ± 0.08	< 0.0001	0.796	34
Overall	0.91 ± 0.03	-0.06 ± 0.02	< 0.0001	0.836	212

Thrum

2	0.64 ± 0.05	0.01 ± 0.04	< 0.0001	0.791	43
4	0.64 ± 0.04	0.007± 0.04	< 0.0001	0.841	47
9	0.54 ± 0.04	-0.08 ± 0.04	< 0.0001	0.827	50
10	0.73 ± 0.05	-0.08 ± 0.04	< 0.0001	0.845	43
13	0.83 ± 0.05	-0.17 ± 0.04	< 0.0001	0.873	36
Overall	0.67 ± 0.02	-0.04 ± 0.02	< 0.0001	0.840	219

Table 5.3 Two-way analysis of variance demonstrating the effect of morph and locality on anther heights and the effect of morph and locality on stigma heights.

Anther Height vs. Bud Length

Source	DF	MS	F Value	P
Morph	1	0.04	21.58	< 0.0001
Locality	4	0.001	0.37	0.828
LogBL	1	8.48	4794.66	< 0.0001
Morph*Locality	4	0.003	1.72	0.144
LogBL*Morph	1	0.40	228.21	< 0.0001
LogBL*Locality	4	0.001	0.65	0.627
logBL*Morph*Locality	4	0.004	2.03	0.090
Error	411	0.002		

Stigma Height vs Bud Length

Source	DF	MS	F Value	P
Morph	1	0.02	2.13	0.145
Locality	4	0.05	6.42	< 0.0001
LogBL	1	17.00	2185.45	< 0.0001
Morph*Locality	4	0.02	2.05	0.087
LogBL*Morph	1	0.42	53.63	< 0.0001
LogBL*Locality	4	0.03	4.09	0.003
logBL*Morph*Locality	4	0.02	1.98	0.096
Error	411	0.008		

Table 5.4 Linear and curvilinear regression analysis of relative growth rate of anther heights against bud length and stigma heights against bud length for pin and thrum floral morphs across five populations of *H. caerulea*. A second order term is included in the analysis.

ANTHER HEIGHT

Pin

Locality	X^2	X	y-intercept	$P(x^2)$	polynomial P(x)	r^2
2	0.08 ± 0.12	0.25 ± 0.21	0.20 ± 0.08	0.507	0.230	0.837
4	-0.01 ± 0.08	0.43 ± 0.14	0.14 ± 0.05	0.939	0.003	0.875
9	-0.004 ± 0.08	0.48 ± 0.14	0.08 ± 0.06	0.960	0.001	0.937
10	-0.02 ± 0.07	0.50 ± 0.11	0.09 ± 0.04	0.761	< 0.0001	0.920
13	-0.002 ± 0.13	0.46 ± 0.23	0.12 ± 0.09	0.988	0.051	0.855
Overall	-0.01 ± 0.04	0.45 ± 0.07	0.12 ± 0.03	0.840	< 0.0001	0.877

Thrum

2	0.22 ± 0.13	0.33 ± 0.22	0.17 ± 0.09	0.102	0.149	0.908
4	0.08 ± 0.08	0.59 ± 0.15	0.09 ± 0.06	0.351	0.0003	0.963
9	0.04 ± 0.08	0.61 ± 0.16	0.10 ± 0.07	0.639	0.0003	0.945
10	-0.02 ± 0.08	0.71 ± 0.12	0.05 ± 0.05	0.802	< 0.0001	0.953
13	0.06 ± 0.08	0.57 ± 0.13	0.11 ± 0.04	0.467	< 0.0001	0.973
Overall	0.09 ± 0.04	0.54 ± 0.06	0.11 ± 0.03	0.019	< 0.0001	0.951

STIGMA HEIGHT

Pin

Locality	X^2	X	y-intercept	$P(x^2)$	polynomial P(x)	r^2
2	0.01 ± 0.23	0.98 ± 0.39	-0.14 ± 0.16	0.962	0.016	0.906
4	-0.37 ± 0.16	1.43 ± 0.26	-0.19 ± 0.10	0.021	< 0.0001	0.887
9	-0.28 ± 0.13	1.39 ± 0.22	-0.24 ± 0.09	0.040	< 0.0001	0.954
10	0.12 ± 0.23	0.69 ± 0.36	0.06 ± 0.13	0.598	0.064	0.789
13	-0.18 ± 0.36	1.31 ± 0.62	-0.33 ± 0.25	0.634	0.042	0.800
Overall	-0.04 ± 0.10	0.97 ± 0.17	-0.08 ± 0.06	0.706	< 0.0001	0.836

Thrum

2	-0.30 ± 0.19	1.13 ± 0.32	-0.20 ± 0.13	0.129	0.001	0.803
4	-0.38 ± 0.15	1.32 ± 0.27	-0.27 ± 0.12	0.014	< 0.0001	0.861
9	-0.48 ± 0.11	1.42 ± 0.20	-0.29 ± 0.09	< 0.0001	< 0.0001	0.879
10	-0.47 ± 0.14	1.49 ± 0.23	-0.34 ± 0.09	0.002	< 0.0001	0.878
13	0.06 ± 0.24	0.73 ± 0.36	-0.14 ± 0.12	0.794	< 0.053	0.874
Overall	-0.43 ± 0.06	1.40 ± 0.11	-0.31 ± 0.04	< 0.0001	< 0.0001	0.868

Table 5.5 Two-way analysis of variance demonstrating the effect of morph and locality on anther heights and the effect of morph and locality on stigma heights with and without a second-order term.

Anther Height vs. Bud Length

Source	DF	MS	F Value	P
Morph	1	0.001	0.39	0.531
Locality	4	0.002	0.88	0.475
LogBL	1	0.16	87.01	< 0.0001
PolyBL	1	0.003	1.84	0.175
Morph*Locality	4	0.0001	0.10	0.984
LogBL*Morph	1	0.003	1.64	0.201
PolyBL*Morph	1	0.002	1.11	0.293
LogBL*Locality	4	0.002	0.93	0.448
PolyBL*Locality	4	0.001	0.75	0.556
logBL*Morph*Locality	4	0.0001	0.05	0.995
PolyBL*Morph*Locality	4	0.0002	0.14	0.969
Error	401	0.002		

Stigma Height vs Bud Length

Source	DF	MS	F Value	P
Morph	1	0.006	0.88	0.348
Locality	4	0.003	0.41	0.801
LogBL	1	0.901	122.35	<0.0001
PolyBL	1	0.091	12.34	0.0005
Morph*Locality	4	0.01	1.78	0.1316
LogBL*Morph	1	0.001	0.08	0.782
PolyBL*Morph	1	0.01	1.82	0.178
LogBL*Locality	4	0.005	0.63	0.639
PolyBL*Locality	4	0.008	1.06	0.376
logBL*Morph*Locality	4	0.01	1.37	0.242
PolyBL*Morph*Locality	4	0.01	1.36	0.248
Error	401	0.007		

addition to the differences in relative growth rates of the bud, variation in the amount of filament elongation can also account for differences in anther heights between the morphs (Riveros et al 1995; Faivre 2000).

Both patterns are found within the family Rubiaceae. Species with anther development similar to *H. caerulea* include: *Guettarda scabra* (Rubiaceae) (Richards and Koptur 1993), *Bouvardia ternifolia* (Rubiaceae) (Faivre 2000), *Primula vulgaris* (Webster and Gilmartin 2006), *Quinchamalium chilense* (Santalaceae) (Riveros et al 1987), and species of *Lithospermum* (Cohen et al. 2008). Intermorph differences in filament height contribute to difference in anther height between the two morphs in *Hedyotis salzmanii* (Rubiaceae) (Riveros et al. 1995), *Palicourea padifolia* (Hernadez and Ornelas 2007) and some species of *Psychotria* (Rubiaceae) (Faivre 2000). Because *Hedyotis caerulea* and one of its congeners, *H. salzmannii*, appear to have different patterns of anther development, the distylous conditions that currently exist in these two species may have arisen independently.

The two *Hedyotis* species also differ in their patterns of stigma development supporting an independent evolution of distyly in *Hedyotis*. In *H. caerulea*, a visual inspection of the plots for stigma heights vs. bud length in thrums showed that, as the bud continues to grow, elongation of the stigma slows down or possibly levels off, producing a curvilinear growth pattern described by the second-order equation. In pins the stigma elongates at a constant rate as the bud grows, resulting in a linear pattern of development. In *H. salzmannii* (Riveros et al 1995), however, as well as in *Bouvardia ternifolia* (Faivre 2000), differences in stigma height between morphs arises from uniform variation in rates of stylar growth during development similarly to the pattern of differences that

contributed to variation in pins found here. The complex development of *H. caerulea* is not unique, however, as a similar pattern was also observed in *Guettarda scabra* (Richards and Koptur 1993) in which thrum stylar growth merely decreases, and in species of *Lithospermum* (Cohen et al 2008) where thrum stylar growth ceased.

CHAPTER VI

SUMMARY AND FUTURE RESEARCH

In order to assess the evolutionary stability of distyly in *Hedyotis caerulea*, two questions need to be answered to a reasonable degree. (1) Is this species truly distylous? In other words, does the species display the entire suite of traits, both structural and functional, that are characteristic of distyly? And, (2) is it still possible for natural selection to act upon the genetic variation that exists in populations of *H. caerulea*, thus allowing further evolution to occur?

Concerning the first question, a distylous species has three signature characteristics: stigma/anther dimorphism with reciprocal herkogamy, a physiologically enforced self-incompatibility system, and ancillary floral polymorphisms (Ganders 1979). Population sampling, measurement of stigmas and anthers of flowers collected, and morphometric analysis of the data indicate the presence of two distinct floral morphs in which the average stigma height of one morph is roughly equal to the anther height of the other, and the average anther height of the first morph is equal to the stigma height of the second (i.e., they show reciprocal herkogamy). The classical interpretation of the adaptive significance of reciprocal herkogamy is that it allows the placement of pollen on

the body of insect pollinators in the positions that promote outcrossing and minimize pollen waste to illegitimate crosses. Quantitative analysis of floral measurements suggests that the heights of stigmas and anthers in *H. caerulea* afford a degree of reciprocal herkogamy within a range displayed by other distylous species.

While attempts in this study to directly measure the degree of self-incompatibility in this species failed, nearly all populations surveyed were isoplethic. This observation is consistent with populations in which mating is disassociative and individuals are highly self-incompatible. Thus, it appears that the pin and thrum floral morphs comprise distinct mating types. Further, it suggests that both self- and intramorph fertilization are largely prevented, in favor of cross-fertilization. Since no significant differences were found between the morphs in paternal fitness (as measured by production of pollen) and maternal fitness (as measured by production of seed), no further gender specialization is expected.

As is characteristic of distylous species, *H. caerulea* displayed several distinct ancillary floral polymorphisms, including shape of corolla tube, papilla size, and pollen size. Morphometric analysis indicated that these differences were highly significant. Thus, *H. caerulea* possesses the third defining trait of distyly, which confirms that distyly in *H. caerulea* is complete with no existing proclivity towards selfing or dioecy.

However, is this mating system stable from an evolutionary perspective? Since natural selection can only act on genetically variable traits, this species must have a degree of variation in its fitness traits, such as size and position of its reproductive organs. Statistical analysis of the measurements of each of the floral characters investigated revealed a significant, and often considerable, degree of variation among

populations. Because of the relatively homogenous environmental conditions of the localities sampled, a major source of that variation is most likely genetic. Characterization of the patterns of early floral development in this species indicated that it differs from at least one of its congeners in this regard, indicating independent evolutionary events led to distyly in *H. caerulea*. Therefore, as shown in *H. caerulea*, distyly appears to be a highly stable mating system, capable of being modified by natural selection as the need arises.

While bluets are delicate plants that live above ground only seven or eight weeks per year, two characteristics of this species help explain its apparent success. When environmental conditions are conducive to growth, bluets display a significant amount of clonal growth through rhizomes which very quickly increases the number of flowers capable of sexual reproduction. Secondly, bluets are perennials. When environmental conditions are unfavorable, many of the plants remain dormant until the next favorable growing season. Light appears to be a significant environmental factor regulating the number of plants flowering each year. As ephemeral flowers, they tend to bloom in the spring before other plants have grown enough foliage to compete with the bluets for sunlight. Once they can no longer successfully compete for sufficient light, the flowers completely die back until the following spring.

Even after conducting this dissertation research, *H. caerulea* should still be considered an understudied species. Its range includes most of eastern North America. Expansion of this study could simply involve broadening the geographic range. The focus could also be broadened from a single species to the entire genus. Several congeners are endemic to North America, some of which are not distylous. Comparisons

among other *Hedyotis* species would allow the testing of hypotheses concerning the evolution of distyly and correlated changes in floral morphology. If greenhouse populations could be established, controlled pollination programs could be conducted to further characterize the degree of self-incompatibility with this species. Molecular studies of the genetic basis of distyly and its mechanism of self-incompatibility are in their infancy. *H. caerulea* could be explored as a potential model for such approaches.

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